Attorney's Docket No.: 029996-0278721
Serial No.: 09/804,409

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Kieffer et al.	Art Unit :	1633
Serial No.	:	09/804,409	Examiner :	Kelly, Robert M.
Filed	:	03/12/2001		
Title	:	COMPOSITIONS AND METHODS FOR REGULATED PROTEIN EXPRESSION IN GUT		

Assistant Commissioner of Patents
Washington, DC 20231

DECLARATION OF DR. ANTHONY CHEUNG
UNDER 37 C.F.R. §1.132

Sir:

1. I, ANTHONY CHEUNG, Ph.D., declare and say I am a resident of Vancouver, British Columbia, Canada. My residence address is: 1127 E. 16th Ave., Vancouver, BC, Canada V5T 4M4. I received Bachelor of Science degree in Biochemistry from the University of British Columbia in 1993. I received a Doctor of Philosophy degree in Physiology from the Tulane University in 1999. I am currently Chief Scientific Officer of enGene, Inc., in Vancouver, BC, Canada. My curriculum vitae has been previously submitted, which reflects my expertise in the areas of molecular biology and biochemistry.

2. I am an inventor of the subject matter claimed in United States Patent Application Serial No. 09/804,409, filed March 12, 2001.

3. I have reviewed the claims that are presently under examination.

4. I understand that claims 31, 34 to 36, 40, 43, 47 to 49, 51, 52, 54, 55, 71 to 73, 76, 78 to 80, 82, 83 and 85 to 88 have been rejected due to an alleged lack of adequate written description for subsequences or variants of chromagranin A promoter. I also understand that claims 31, 34 to 36, 38 to 40, 43, 47 to 49, 51 to 55 and 87 have been rejected due to an alleged lack of enablement for any diabetes treatment or onset, animal, chromagranin A promoter, mucosal endocrine cell, polypeptide, amino acid, fat, production of insulin, transformation method, vector, method of nutrient contacting. I further understand that claims 71 to 73, 76, 78 to 80, 82, 83, 85, 86 and 88 have been rejected due to an alleged lack of enablement to treat the body mass or obesity or onset.

5. I submit this declaration and refer to studies published as of the filing of Application Serial No. 09/804,409, and accompanying Exhibit A, a summary diagram of chromagranin A promoter as evidence that functional chromagranin A promoter subsequences and variants would be known to the skilled artisan.

6. I further submit this declaration and refer to studies herein and in accompanying Exhibit B as evidence that other vectors, including non-viral vectors, can effectively transform gut or gastrointestinal cells *in vivo*, and that the transformed cells can produce therapeutic levels of insulin or leptin in the animal.

7. I also submit this declaration and refer to studies herein and in accompanying Exhibit C as evidence that gut or gastrointestinal endocrine cells other than K cells can process proinsulin to bioactive insulin and, therefore, can produce bioactive insulin.

8. I additionally submit this declaration to refer to studies by others in accompanying Exhibit D reporting that leptin is effective in treating an animal model of human obesity caused by leptin resistance.

9. ***Chromagranin A Promoter Structure and Function, Exhibit A***

Exhibit A, submitted herewith, is a diagram summarizing knowledge in the art of chromagranin A promoter structure and function at the time of the invention. In brief, the promoter sequence region from –4800 to –2200 relative to the coding sequence has been reported to contain a positive domain, and a negative domain between –258 and –181 (see, Wu et al., J. Clin. Invest. 94:118 (1994)). The promoter sequence region between –726 and –455 contains a 27 base pair AP-1 binding sequence (-576 to –550) that has been reported to enhance promoter activity about 10-fold (see, Nolan et al., Endocrinol. 136:5632 (1995); and Nolan et al., Mol. Cell. Endocrinol. 124:51 (1996)). A CRE enhancer has been reported at –147 to –100 (see, Wu et al., J. Clin. Invest. 96:568 (1995)). An Sp-1/Egr-1 site spans –88 to –77, and a cyclic AMP response element (CREB) spans 71 to –64, which have been reported to mediate gastrin activation of the promoter (see, Wu et al., J. Clin. Invest. 96:568 (1995)). A "TATA" box is present from -22 to –18, and a glucocorticoid response element (GRE) located between –583 and –597 of chromagranin A promoter was reported to confer glucocortocid responsiveness

(Rozansky et al., J. Clin. Invest. 94:2357 (1994)). Most of these Chromagranin A promoter regions have been reported or are believed to be functionally conserved between rat, mouse and human.

In addition to various functional regions of Chromagranin A promoter, promoter sequences exhibit significant sequence homology. For example, mouse and rat chromagranin A promoter share 85% homology and mouse chromagranin A promoter shares 52% homology with the bovine promoter. Two regions of mouse and human chromagranin A promoter, from -1107 to -1040 and from -5752 to -5475 relative to the putative transcription start site, share 78% and 85% identity, respectively.

10. Based upon the foregoing knowledge of chromagranin A promoter structure and function, chromagranin A promoter functional conservation and chromagranin A promoter sequence homology at the time the application was filed, I conclude that functional chromagranin A promoter subsequences and variants would be known to the skilled artisan.

11. ***Non-viral Vector Transformation of Gut or Gastrointestinal Cells In Vivo and Production of Therapeutic Levels of Insulin in Animals, Exhibit B***

Studies indicating that *in vivo* transformation can be performed with other vectors, including non-viral vectors, and that the transformed cells produce therapeutic levels of insulin in animals were performed as follows:

Formation of chitosan-DNA nanoparticles: Chitosan (Molecular Weight: ~ 5.2 kD; degree of deacetlyation: 98%) was obtained from Biosyntech (Montreal, Canada). The plasmids used include an expression plasmid for ϕC31 integrase (pCMVInt) and a targeting plasmid containing the GIP promoter linked insulin gene (pGIP/Ins-attB). The pGIP/Ins-attB plasmid consists of the attB recognition site for genome integration by the ϕC31 integrase (Olivares et al. Nature Biotechnology 20:1124 (2002)). The pCMVInt and pGIP/Ins-attB were packaged at a ratio of 1:5 (w/w) in chitosan polymer at a N:P ratio of 60:1.

Plasmid and chitosan solutions, prepared separately, were adjusted to a concentration equal to 2x the required final concentration. Plasmid was diluted in water. The indicated chitosan was dissolved in 5mM sodium acetate, pH5.5. Both solutions were incubated at 55°C for 5 minutes before being mixing. Equal volumes of the two solutions were mixed and rapidly

vortexed for 30 seconds to form plasmid/chitosan particles. For certain applications, this preparation was further diluted in various buffers prior to analysis.

Production of FIV and various AAV vector particles: Recombinant feline immunodeficiency virus (FIV) vector particles were packaged by co-transfecting 293T cells with an established triple-plasmid calcium phosphate transfection protocol. Polynucleotides encoding the proteins were an FIV vector harboring a human insulin gene that was driven by a rat GIP promoter. The other two plasmids were 1) an FIV packaging construct expressing FIV gag and pol genes; and 2) an envelope construct expressing VSV-G envelope glycoprotein. Briefly, 293T cells ($1.5x10^7$) were transfected with 8 μg of packaging plasmid, 4 μg of envelope plasmid and 8 μg of vector plasmid in each 150 mm cell culture dish. Eight hours after transfection, cells were fed with fresh media (Dulbecco Modified Eagle media with 10% FBS) and incubated at 37°C overnight. The culture media was replaced the next morning, transfected cells were transferred to a 32°C incubator and virus particles were harvested from the culture media at 48 hours and 72 hours post-transfection, concentrated by centrifugation at 50,000xg for 2 hours and stored in TNE buffer (50 mM of Tris and 130 mM of NaCl and 1mM of EDTA) at -80°C until use. FIV vector titers were determined by real-time quantitative PCR (RQ-PCR) using primers and fluorescent probes specific for the gene-of-interest. A9L mouse fibroblast cells ($5x10^5$) were infected with 5 μl of FIV virus and the genomic DNA of infected cells isolated 48 hours later with a DNAeasy Tissue Kit (Qiagen, Chatsworth, CA).

For AAV vector production, an established adenovirus-free, three-plasmid co-transfection method was used. To produce AAV vector, the vector plasmid carrying the GIP promoter linked insulin gene, packaging plasmid (pAAV-RC for AAV-2 vectors) and mini-Ad helper plasmid (pHelper) were co-transfected into 293 cells. In detail, human 293 cells were passaged into thirty 150 mm tissue culture dishes. A total of 30 μg plasmid DNA (10 μg of each plasmid) were transfected using a calcium phosphate precipitation method as described previously. Eight hours post transfection, the media was replaced with fresh Dulbecco modified Eagle media (DMEM) with 10% FCS and antibiotics. At 48 hours post-transfection, the cells were harvested by scraping and low speed (1500 rpm) centrifugation

for 15 minutes. The cell pellet was resuspended in 20 ml of TMN (20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 150 mM NaCl). After 2 cycles of freeze/thaw in dry ice/ethanol bath, deoxycholic acid and Benzonase (Sigma) were added to the cell lysate to a final concentration of 0.5% and 50 U/ml respectively and incubated for 30 minutes at 37° C. The cell debris was removed by centrifugation at 2000xg for 15 minutes at 4°C, the supernatant clarified by incubation at 56°C for 45 minutes and stored at –20°C overnight. AAV vectors were purified by thawing the lysate at 37°C, spinning at 2000xg for 30 minutes at 4°C to remove the flocculent precipitate, filtering the supernatant through a 0.8μm filter, and passing through a HiTrap Heparin HP column. AAV vectors were eluted from the column with elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 M NaCl) and put through a HiTrap Desalting column to exchange the elution buffer to TMN.

Delivery of FIV, AAV vectors and chitosan-DNA particles to Duodenum of mice: Male C57/BL6 mice were housed in a 12 hour light/dark cycle. Animals were fasted overnight prior to surgical procedure for vector delivery. Animals were anesthetized by continuous isofluorane inhalation. An abdominal incision was made and a section of the duodenum (~2 cm) was lifted from the abdominal cavity using a glass hook. A tourniquet was placed around the pyloric sphincter and the isolated section of the duodenum was washed once with warm saline followed by a 10 minute incubation with ~0.12 ml of 0.2% n-Dodecyl-beta-D-maltoside (DDM). The DDM was then washed twice with ~150 μl of warm saline and the isolated section of the duodenum was maintained in an elevated position. The viral vector was delivered to the lumen of the duodenum by a single injection, which was allowed to incubate in the elevated duodenum section for 1 hour. After incubation, the tourniquet was released, the duodenum returned to the abdominal cavity and the incision closed.

Measurement of Gene Delivery to Duodenum: The amount of DNA transfected into duodenal tissue was measured by quantitative polymerase chain reaction (Q-PCR). Tissue samples were thawed to room temperature then ground with a pestle and mortar. DNA was extracted using the manufacturer's instructions for the Qiagen DNeasy Tissue Kit, except that double the listed volumes of proteinase K and ATL buffers were used. Genomic DNA concentrations were measured using a spectrophotometer prior to amplification using primers

specific for the particular transgene studied. Standardization was also carried out using primers for 18S RNA coding sequence. The amplification was quantified using blank samples spiked with plasmid DNA. Amplification was carried out using and ABI 7000 cycler.

Blood collection and measurement of human C-peptide levels: Blood samples were collected in heparinized capillary tubes by saphenous vein bleeding and transferred to a microcentrifuge tube containing 3µL Trasylol. Plasma was isolated from the blood samples by centrifugation and stored at –80°C until assayed. Plasma levels of human C-peptide (a by-product of insulin biosynthesis) were measured using a commercially available ELISA kit (Alpco). The antibodies used in the ELISA assay specifically recognize the C-peptide of human origin and minimally cross react (0.08%) with mouse C-peptide. Assays were performed according to the manufacturer's instructions.

Results: Two weeks after vector administration, gene copies in duodenal mucosa were compared. Six of seven viral vectors, namely AAV2, AAV5, AAV6, AAV1, AAV2.5 and FIV, were able to deliver genes into gut mucosal tissue at comparable levels (Exhibit B, panel A). The chitosan-based vector delivered genes into the duodenal mucosa at a higher efficiency compared to FIV and AAV vectors, indicating that the polymer-based non-viral vector system appears more efficient than FIV and AAV vectors in delivering genes to the intestine (Exhibit B, panel A). In any event, the observation that the transformed genes were present in mucosal tissue even after 14 days indicates that the vector consistently integrated the transgene into a stem or precursor cell population -the life span of differentiated epithelial cells of the duodenum is estimated to be only 3-5 days.

Administration of non-viral vector to duodenum of mice resulted in long term production of therapeutic levels of insulin in the bloodstream. As shown in Exhibit B, panel B, a single administration of chitosan-packaged plasmids resulted in continuous production of human C-peptide for over 115 days following administration. Furthermore, secretion of human C-peptide was inducible by oral glucose administration to these animals (Exhibit B, panel C). The glucose-inducible secretion profile in the animals further indicates that the human insulin transgene was targeted to appropriate cells, such as K-cells.

These results indicate that administration of chitosan encapsulated plasmid DNA, containing the GIP promoter-linked human insulin gene, to lumen of mice duodenum, resulted in systemic long-term production of orally glucose-inducible human insulin at therapeutic amounts. These results are consistent with results obtained for viral vectors (e.g., AAV-2 and FIV) which can also effectively transfer genes into gut or gastrointestinal mucosa *in vivo* for expression of encoded protein at therapeutic levels.

12. Based upon the foregoing results and the results of record, I conclude that viral and non-viral vectors can be used to deliver insulin or leptin gene into gut or gastrointestinal cells *in vivo* to produce transformed cells without undue experimentation. I also conclude that 1) transformed cells can exhibit long term production of the insulin or leptin in animals; 2) insulin or leptin can be produced at levels sufficient to treat animals, indicating that therapeutic levels can be produced; and 3) insulin or leptin can be produced in response to glucose and other nutrients.

13. ***Gut or Gastrointestinal Cells other than K Cells Produce Insulin, Exhibit C***

STC-1 cells comprise a mixed population of entero-endocrine cells (Rindi et al., Am. J. Pathol. 136:1349 (1990)). STC-1 cells include S-cells, which produce secretin (Rindi et al., Am. J. Pathol. 136:1349 (1990); and Wheeler et. al, Mol. Cell. Biol. 12:3531 (1992)); L-cells, which produce proglucagon related peptides (Rindi et al., Am. J. Pathol. 136:1349 (1990); Tucker et al., Regul. Pept. 62:29-35 (1996); Abello et al., Endocrinology 134:2011 (1994); and Litvak et al., J Gastrointest. Surg. 3:432 (1999)); K cells, which produce GIP (Rindi et al., Am. J. Pathol. 136:1349 (1990); Boylan et al., J. Biol. Chem. 272:17438 (1997); and Steinhoff et al., Regul. Pept. 97:187 (2001)); D-cells, which produce somatostatin (Rindi et al., Am. J. Pathol. 136:1349 (1990); and Gajic et al., Endocrinology 132:1055 (1993)); cholecystokinin producing cells (Gajic et al., Endocrinology 132:1055 (1993); Lay et al., Am. J. Physiol. Gastrointest. Liver Physiol. 288:G354 (2005); and Guilmeau et al., Diabetes 52:1664 (2003)); and neurotensiin producing cells (Rindi et al., Am. J. Pathol. 136:1349 (1990)).

Studies demonstrating that cells other than K cells produce insulin were performed as follows:

Cell culture and transfection: STC-1 cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 25 mM glucose and 10% fetal calf serum. Twenty-four hours prior to transfection, STC-1 cells were seeded into a 6-well plate at a density of $0.3x10^6$ cells per well. STC-1 cells were transfected with 1 µg of a plasmid carrying the CMV promoter-linked human insulin construct using FuGENE 6 Transfection Reagent (Roche) according to manufacturer's protocol. The CMV promoter is a constitutive promoter having activity in the various gut endocrine cells present in STC-1 cells. The STC-1 cell line is a mixed population of immortalized entero-endocrine cells (K-cells, L-cells, S-cells, G-cells and other types of entero-endocrine cells).

Immunohistochemistry: Transfected STC-1 cells were then immunostained with antibodies specific for insulin and GIP as follows: Forty-eight hours after transfection, STC-1 cells were washed thrice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Fixed cells were then permeabilized by incubation in PBS containing 0.3% Triton X-100, 1% PFA for 5 minutes at room temperature. Following overnight blocking in PBS with 5% normal goat serum (NGS) and 1% BSA at 4°C, cells were incubated for 90 minutes at room temperature in blocking buffer containing antibodies specific for GIP and human insulin (BioDesign, E54071M) at a titer of 1:200 and 1:100, respectively. The cells were then washed three times in PBS, followed by incubation in PBS containing appropriate fluorescence-conjugated secondary antibodies (titer=1:800) for 30 minutes at room temperature. The cells were then washed thrice in PBS, treated with Vectashield H-1200 and examined with a fluorescence microscope.

Results: The ability of non-K-cell gut endocrine cells to produce bioactive insulin was determined by staining transfected STC-1 cells with antisera specific for human insulin and mouse GIP. As expected, immunoreactivity to human insulin protein was detected in K-cells that express GIP, indicating that K-cells can process proinsulin into bioactive insulin (Exhibit C, panel A). In addition, clusters of non-K gut endocrine cells within the transfected STC-1 cells (non K cells do not stain for GIP) are immunoreactive for bioactive insulin (Exhibit C, panel B).

14. Based upon the foregoing studies, I conclude that gut or gastrointestinal endocrine cells other than K-cells can process proinsulin into bioactive insulin. Given that endocrine cells other than K-cells can produce bioactive insulin, I predict that these other endocrine cells will secrete bioactive insulin in response to a sugar, polypeptide, amino acid or fat.

15. ***Leptin Treatment of Leptin Resistant Animals Reduces Obesity, Exhibit D***

Studies indicating that leptin can be used to treat animals resistant to leptin are reported in a publication by Orci et al. (Proc. Natl. Acad. Sci. USA 101:2058 (2004), submitted herewith as Exhibit D). In brief, the authors describe studies in which Zucker rats were administered an adenovirus vector bearing leptin cDNA driven by a CMV promoter intravenously (Exhibit D, page 2058, Experimental Procedures, and page 2059, Results). Zucker rats have a mutant leptin receptor that impairs leptin binding and downstream action, resulting in obesity (see, for example, Wang et al., Proc. Natl. Acad. Sci. USA 95:714 (1998); and Wang et al., Proc. Natl. Acad. Sci. USA 95:11921 (1998)). The authors report that 1) body weight in the treated Zucker rats declined; and 2) plasma leptin levels increased in rats following administration of the vector bearing leptin cDNA (Exhibit D, page 2059, Results).

16. Based upon the foregoing results, I conclude that leptin would be an effective treatment of animals that are resistant to leptin.

17. ***Summary of Conclusions***

In view of the foregoing studies and supporting publications, and the previous studies and supporting publications of record, I make the following conclusions:

A. In view of the knowledge in the art regarding chromagranin A promoter structure and function, functional chromagranin A promoter subsequences and variants would be known to the skilled artisan;

B. Gut or gastrointestinal endocrine cells other than K cells can process proinsulin to bioactive insulin thereby producing bioactive insulin in animals;

C. Various vectors, including viral and non-viral vectors, can be used to transform gut or gastrointestinal cells *in vivo*, to an extent that transformed cells produce therapeutic levels of encoded protein (e.g., insulin or leptin) in the animal; and

D. Leptin would be effective in treating leptin resistant mammals, such as resistance caused by a mutated leptin receptor.

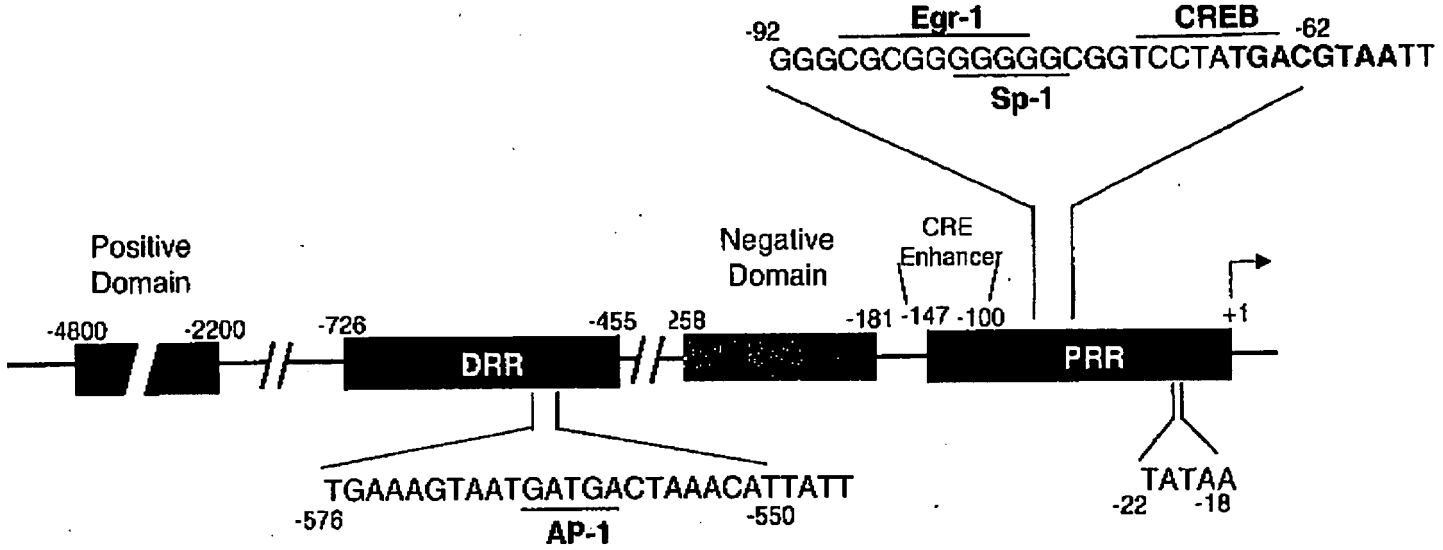
18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

June 20th, 2006
Date

ANTHONY CHEUNG, Ph.D.

EXHIBIT A

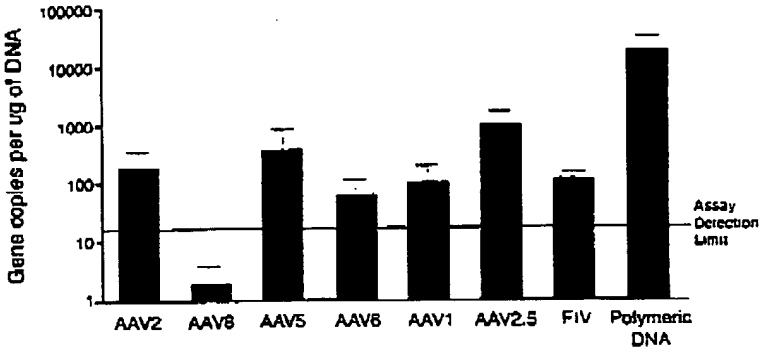
Functional Domains Within the Endocrine Cell-specific Promoter of Chromogranin A



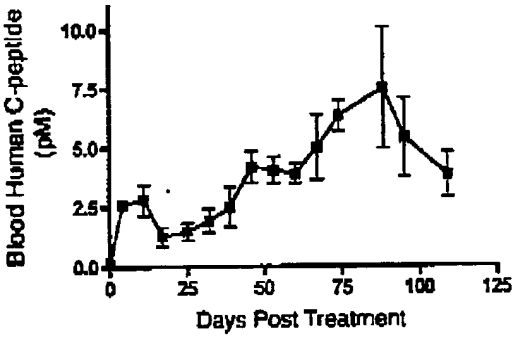
- + Other DRR binding proteins
- Enhanced activity by 10X

References:
Wu H., et. al. J Clin Investigation, 1994, 94:118-129
Wu H, et. al. J Clin Investigation, 1995, 96:568-578
Nolan EM, et. al. Mol Cell Endocrinol,. 1996, 124: 51-62
Nolan EM, et. al. Endocrinology, 1995: 136(12):5632-5642

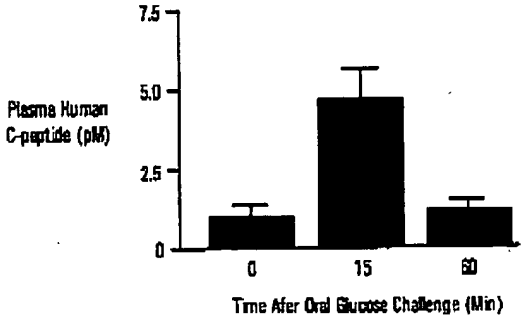
Exhibit B



Panel A: Numbers of marker gene copies in duodenal mucosal tissue. Different types of viral vectors and chitosan polymer-based non-viral vector were compared. An equal volume of vector solutions were injected into the lumen of the duodenum. Values are mean ± SEM. n=4 to 6 per group.

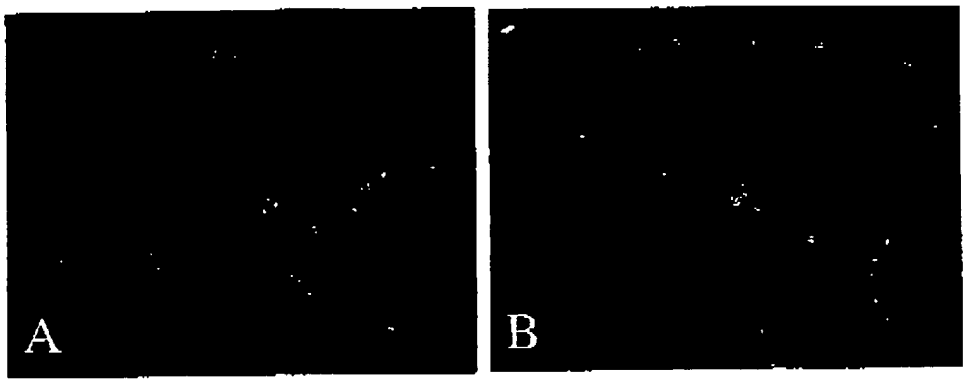


Panel B: Plasma human C-peptide levels in mice treated with chitosan-packaged rGIP/hIns gene. Two hundred and fifty µl of vector solution was incubated in the lumen of the duodenum of C47/BL6 mice (n=6) as described. Data shown are mean ± SEM.



Panel C: Oral glucose dependent secretion of human C-peptide in mice treated with chitosan-packaged rGIP/hIns gene. Animals were fasted overnight and a glucose bolus (2 g/kg dose in 200 µL volume) was given by oral gavage. Blood samples were collected at times indicated. Data shown are mean ± SEM.

Exhibit C



Immunohistochemical staining for mature human insulin in a gut endocrine cell line (STC-1). Cultured STC-1 cells were transfected with a CMV-promoter linked human proinsulin gene construct, followed by co-staining with antisera specific for mature insulin (red) and GIP (green). Cells were also stained with DAPI to demarcate the position of nuclei in cells (panel A). Immunoreactivity to mature human insulin was detected in cluster of GIP-expressing K-cells (panel B). Representative micrograph depicting a distinct cluster of non-K-cells (negative for GIP immunoreactivity, non-green cells) capable of expressing mature human insulin.

EXHIBIT D

Rapid transformation of white adipocytes into fat-oxidizing machines

Lelio Orci*†, William S. Cook‡, Mariella Ravazzola*, May-yun Wang‡, Byung-Hyun Park‡, Roberto Montesano*, and Roger H. Unger†‡§

*Department of Morphology, University of Geneva Medical School, Rue Michel Servet 1, CH 1211 Geneva 4, Switzerland; ‡Gifford Laboratories of the Touchstone Center for Diabetes Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Y8.212, Dallas, TX 75390-8854; and §Veterans Affairs Medical Center, Dallas, TX 75216

Contributed by Roger H. Unger, December 15, 2003

Adenovirus-induced hyperleptinemia rapidly depletes body fat in normal rats without increasing free fatty acids and ketogenesis, implying that fat-storing adipocytes are oxidizing the fat. To analyze the ultrastructural changes of adipocytes accompanying this functional transformation, we examined the fat tissue by electron microscopy. After 14 days of hyperleptinemia, adipocytes had become shrunken, fatless, and encased in a thick basement-membrane-like matrix. They were crowded with mitochondria that were much smaller than those of brown adipocytes. Their gene expression profile revealed striking up-regulation of peroxisome proliferator-activated receptor γ coactivator 1α (an up-regulator of mitochondrial biogenesis not normally expressed in white fat), increased uncoupling proteins-1 and -2, and down-regulation of lipogenic enzymes. Phosphorylation of both acetyl CoA carboxylase and AMP-activated protein kinase was increased, thus explaining the increase in fatty acid oxidation. The ability to transform adipocytes into unique fat-burning cells may suggest novel therapeutic strategies for obesity.

Intense hyperleptinemia induced in normal rats by means of adenovirus-mediated transfer of the leptin gene causes the rapid disappearance of all visible body fat within 7 days (1). Because this fulminating fat loss is unaccompanied by any increase in plasma free fatty acids (FFA) and β-hydroxybutyrate levels or of ketonuria (2), it contrasts sharply with the fat loss induced by starvation and insulin deficiency, in which FFA released from adipocytes are oxidized to ketones in the liver (3). A possible explanation for this radical metabolic difference is that in the hyperleptinemic animals FFA are oxidized inside the adipocytes. The gene expression profile of overleptinized adipose tissue is consistent with this hypothesis (4), as is the fact that isolated adipocytes respond to recombinant leptin with a release of glycerol unaccompanied by the parallel release of FFA induced by other lipolytic hormones (5).

In this article, we report on the morphologic and molecular changes that occur in white adipocytes as they are transformed by hyperleptinemia into mitochondria-rich fat-burning cells. The findings suggest a metamorphosis of fat-laden white adipocytes into a unique fat-free, mitochondria-rich cell type morphologically distinct from other related cells. The remarkable increase in internal combustion of fat seems to result from a leptin-induced increase in the phosphorylation state of AMP-activated kinase (AMPK), together with increased expression of peroxisome proliferator-activated receptor (PPAR)γ coactivator 1α (PGC-1α) and thermogenic proteins and decreased expression of lipogenic enzymes.

Experimental Procedures

Animals. Eight-week-old wild-type Zucker diabetic fatty (ZDF) rats (+/+) weighing between 280 and 300 g were used. Adenovirus containing either the rat leptin cDNA (AdCMV-leptin) or the β-galactosidase cDNA with the cytomegalovirus (CMV) promoter was prepared and administered intravenously as described (1). Body weight measurements confirmed weight loss. Hyperleptinemia was quantified by leptin RIA of plasma (Linco Research Immunoassay, St. Charles, MO). Tri-iodothyronine was measured with the DiaSorin Free T_3 RIA kit (Stillwater, MN). Rats were killed under pentobarbital anesthesia at 7, 10, or 14 days after injection. An epididymal fat pad or its remnant was resected from each rat and fixed for microscopy or frozen in liquid nitrogen for mRNA or protein analysis.

Processing for Light and Electron Microscopy. Epididymal fat pads and fat pad remnants from hyperleptinemic and control animals were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). For conventional electron microscopy, the samples were postfixed with OsO_4, dehydrated with alcohol, and embedded in Epoxy embedding medium (Epon; Fluka). For light microscopy immunohistochemistry, the OsO_4 step was omitted. For electron microscopy immunocytochemistry, samples of glutaraldehyde-fixed epididymal fat were embedded in 12% gelatin, infused with 2.3 M sucrose, and frozen in liquid nitrogen for cryoultramicrotomy. Ultrathin cryosections were cut at −120°C and picked up with a mixture (1:1) of 2.3 M sucrose and 1.8% methylcellulose (6). Semithin (1-μm thick) sections of Epon-embedded fat were examined by phase contrast microscopy. Immunofluorescence reactions were performed on semithin sections of nonosmicated tissue after removal of Epon. Sheep anti-collagen IV (a gift of G. Martin, National Institute of Aging, Bethesda, MD) was used at 1:1,000 dilution, followed by FITC-conjugated rabbit anti-sheep IgG (Biosys, Columbia, MD). Ultrathin cryosections were incubated with sheep antibody to collagen IV (1:2,000 dilution) or rabbit antibody to mouse perlecan (a gift of J. Hassel, University of Pittsburgh, Pittsburgh, PA) diluted at 1:250. The sections were subsequently incubated with rabbit anti-sheep antibody followed by protein A-gold (for anti-collagen IV) or directly with protein A-gold (for anti-perlecan). For quantification of adipocyte size, semithin sections (1- to 2-μm thick) or thin sections were analyzed by using a Zeiss Axiophot microscope equipped with an AxioCam digital camera. Data of sectional area and cell perimeter were obtained by using the AXIOVISION software for digital image processing. Data are presented as mean ± SEM.

Real-Time PCR Analysis of mRNA. To quantify mRNA by real-time RT-PCR, total RNA was prepared from frozen tissue samples by using Trizol according to the manufacturer's protocol (Invitrogen). Genomic DNA was removed from the total RNA preparations by using DNA-free Dnase 1 (Ambion, Austin, TX). RNA from each sample was diluted to 5 ng/μl, and 100 ng of RNA was reverse transcribed in a 100-μl reaction with random hexamers

Abbreviations: AMPK, AMP-activated kinase; PPAR, peroxisome proliferator-activated receptor; PGC-1α, PPARγ coactivator 1α; UCP, uncoupling protein; ACC, acetyl CoA carboxylase.

†To whom correspondence should be addressed. E-mail: roger.unger@utsouthwestern.edu or lelio.orci@medecine.unige.ch

www.pnas.org/cgi/doi/10.1073/pnas.0308258100

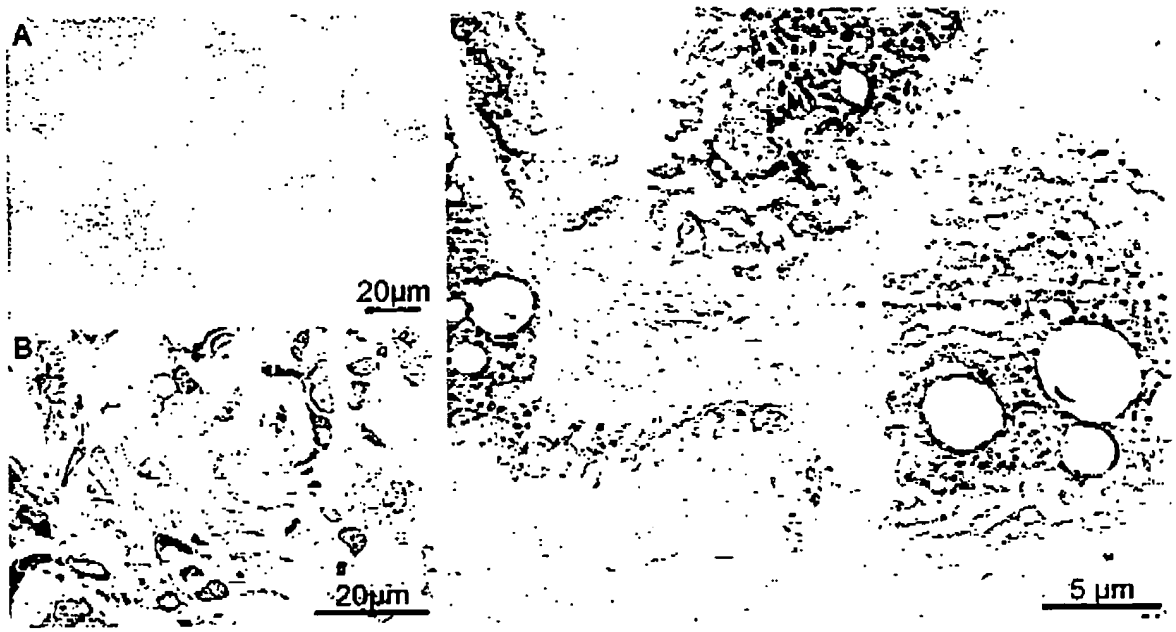


Fig. 1. Hyperleptinemia induces extensive lipid depletion in adipose tissue. (*A*) Semithin section of normal epididymal fat pad tissue from control rats 14 days after infusion with β-galactosidase adenovirus. (*B*) Epididymal fat cells 14 days after infusion with leptin adenovirus. The cells are fat-depleted with a few residual lipid droplets and a highly indented surface. (*C*) Low magnification electron microscopic view showing a highly convoluted surface of postadipocytes embedded within an exceedingly thick layer of apparently amorphous material, which separates individual cells from the collagen-fibril-rich interstitial matrix.

(TaqMan Reverse Transcription kit, Applied Biosystems) as described (7). Data were analyzed by using SDS 2.1 software (Applied Biosystems). Relative quantification of gene expression was by the comparative C_T method with 36B4 mRNA as an endogenous control (User Bulletin no. 2, Applied Biosystems). Primers were designed with PRIMER EXPRESS 2.0 (Applied Biosystems). Their sequences are shown in Table 2, which is published as supporting information on the PNAS web site.

Immunoblotting Analysis. Total cell extracts prepared from fat tissues of lean +/+ rats that were treated with AdCMV-leptin virus for 14 days were resolved by SDS/PAGE and transferred to poly(vinylidene difluoride) membrane (Amersham Pharmacia Biosciences). The blotted membrane was treated in 1× Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk for 1 h at room temperature with gentle, constant agitation. The following antibodies were used: anti-acetyl CoA carboxylase (ACC) (Alpha Diagnostic, San Antonio, TX), anti-AMPKα and anti-phospho-AMPK (Thr-172) (Cell Signaling Technology, Beverly, MA), anti-phospho-ACC (Ser-79) (Upstate Biotechnology, Lake Placid, NY), anti-cytochrome *c* (BD Biosciences/Pharmingen), anti-cytochrome *c* oxidase IV subunit 5b (Molecular Probes), and anti-uncoupling proteins (UCP)-1 and -2 (Alpha Diagnostics).

Results

Clinical Effects of Adenovirus-Induced Hyperleptinemia. Lean wild-type (+/+) Zucker diabetic fatty rats with an average body weight of 280 ± 20 g received recombinant adenovirus containing the leptin cDNA. Body weight declined to 207 ± 5 g in 14 days. Plasma leptin levels rose from 3.5 ± 1.2 ng/ml before treatment to a value of 265 ± 88 ng/ml 7 days later, followed by a decline to 36 ± 12 ng/ml at 14 days. Tri-iodothyronine levels were normal. Hyperleptinemic animals were in their usual state of health, except for a 30% reduction in food intake.

Microscopic Features of Lipid-Depleted Adipocytes. After induction of hyperleptinemia, the epididymal fat pad was rapidly transformed into a fatless, hypervascular fat pad remnant (data not shown). In the place of the well differentiated spheroidal adipocytes present in control rats (Fig. 1*A*), semithin sections of the remnant fat pad revealed irregularly shaped shrunken cells in a well vascularized stroma with extreme folding of the cell surface (Fig. 1*B*). There was a dramatic decrease in the size of leptinized adipocytes when compared with fat cells of control rats: the sectional area of untreated control adipocytes declined in 14 days from 4,147 ± 255 μm^2 to 102 ± 6 μm^2, whereas cell perimeter was proportionally less reduced (from 313 ± 11 μm to 183 ± 20 μm), as expected from the highly indented surface of postadipocytes.

A thick pericellular matrix filled the infoldings of the cell surface (Fig. 1*C*). At higher magnification (Fig. 2*A*), the matrix displayed a texture similar to, albeit less compact than, that of the basal lamina that surrounds well differentiated adipocytes. In contrast to the thin line of collagen IV immunofluorescent staining that surrounds normal adipocytes (8) (Fig. 2*B*), collagen IV immunofluorescence thickened progressively in the leptinized cells (Fig. 2 *C* and *D*).

Although electron microscopy revealed ImmunoGold labeling for collagen IV and perlecan (two major components of basement membranes) to be present throughout the pericellular matrix (data not shown), the mRNAs of collagen IV, perlecan, and laminin α4 and β1 chains were not increased (Table 2) nor was there a reduction in the mRNAs of three matrix metalloproteinases involved in extracellular matrix remodeling (Table 2). However, the expression of the physiological metalloproteinase inhibitors TIMP-1, -2, and -3 (9) was increased at 7 days but not thereafter (Table 2). Expression of β1 integrins, which play an important role in basement-membrane assembly (10), was markedly reduced at 10 and 14 days of hyperleptinemia (Table 2), raising the possibility that β1 integrins were insufficient for the formation of a normal basal lamina.

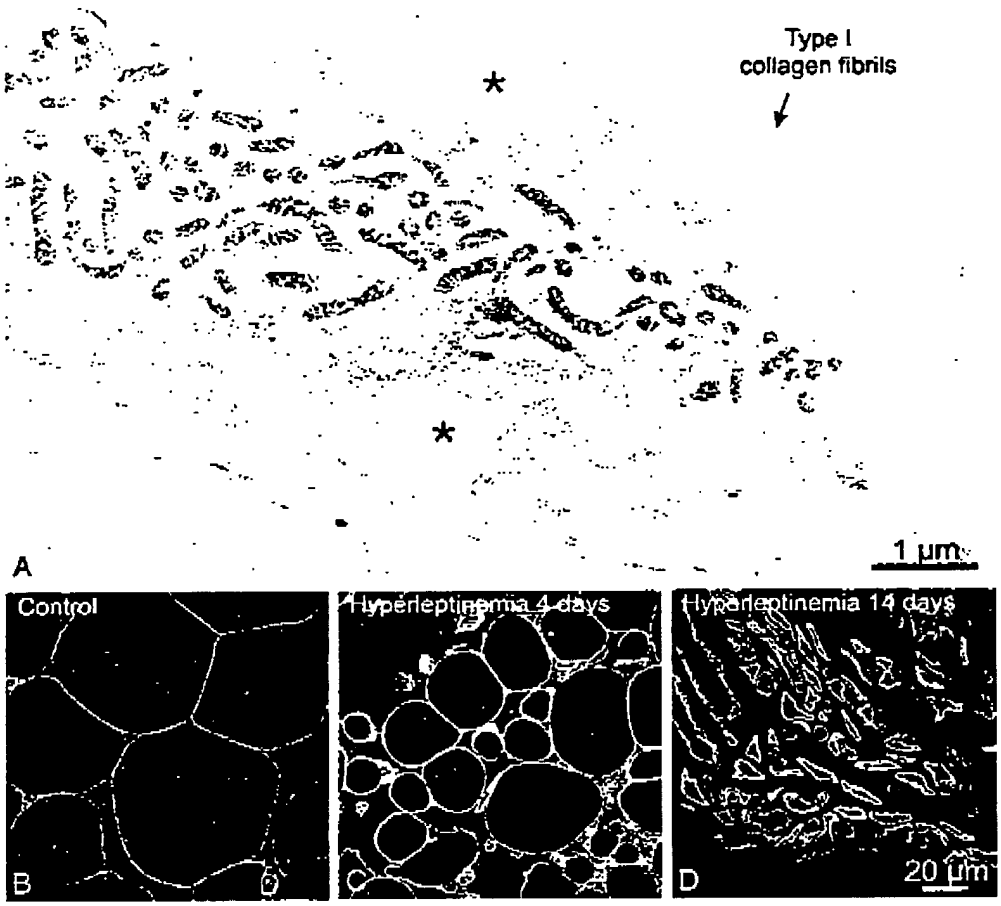


Fig. 2. Ultrastructural and immunocytochemical features of the postadipocyte pericellular matrix. (*A*) Postadipocytes from rats infused for 14 days with leptin adenovirus are surrounded by a very thick layer of finely granular material (asterisks) that separates them from the collagen fibrils of the interstitial matrix. (*B*) Immunocytochemical localization of collagen type IV in control epididymal fat pad tissue. Immunoreactivity is detected as a thin, faint line surrounding each individual adipocyte and corresponding to the location of the basal lamina. (*C*) Four days after the infusion of leptin adenovirus, adipocytes still present a spheroidal shape, but their size has decreased. Collagen IV staining at the cell periphery appears considerably thicker than in control rats. (*D*) After 14 days of hyperleptinemia, adipocytes are further reduced in size, and their highly indented surface displays a thick, scalloped collagen IV immunoreactivity.

Expression of Adipocyte Cell Markers. To determine whether the lipid-depleted white adipocytes had been converted to a closely related fat-burning cell type, brown adipocytes, we assessed the protein or mRNA of genes that are normally expressed at high levels in those cells. UCP-1, normally found only in brown adipose tissue, was present by immunoblotting in the white fat of the leptinized rats (Fig. 3*D*), but it was well below the levels normally present in brown adipose tissue. Cidea mRNA, which is present in brown adipocytes (11), was not detected (Fig. 3*A*). The molecular hallmarks of mature white adipocytes, such as adipocyte fatty acid-binding protein 2 (aP_2) and leptin, had disappeared (Fig. 3*A*), but some brown adipocytes markers, such as Cidea, were absent. For this reason they were designated postadipocytes.

Expression of Transcription Factors of Lipid Metabolism. Neither PPARα, which targets enzymes of fatty acid oxidation and is necessary for the fat-depleting action of hyperleptinemia on adipocytes (12), nor PPARδ, recently shown to activate fat burning (13), were altered by hyperleptinemia. The adipogenic transcription factors SREBP-1c, ChREBP, and FOXO1 (14), but not PPARα, were significantly suppressed during the first week of hyperleptinemia, whereas the anti-adipogenic factor FOXC2 (15) was significantly increased (Table 1).

Activity and Expression of Enzymes of Fat Metabolism. Conversion of adipocytes from fat-storing to fat-burning cells could be the result of a reduction in malonyl CoA, the substrate for lipogenesis and an inhibitor of fatty acid oxidation (16). Because malonyl CoA production can be blocked by phosphorylation of ACC (17), we measured P-ACC (α plus β) in the fat pad remnant and found it to be increased compared to unleptinized control rats (Fig. 3*B*). Because ACC phosphorylation is catalyzed by AMPK, which is activated by phosphorylation (17, 18), we also measured P-AMPK. It, too, was increased in the fat tissue of leptinized rats (Fig. 3*B*), confirming the finding of Minokoshi *et al.* (19). It is likely that the increase in P-AMPK, by inactivating ACC, was a factor in the lipid depletion in adipocytes.

To determine the role of down-regulation of expression of lipogenic enzymes, we measured the mRNAs of ACCα and ACCβ, fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD-1), and glycerol-3-phosphate acyl transferase (GPAT)

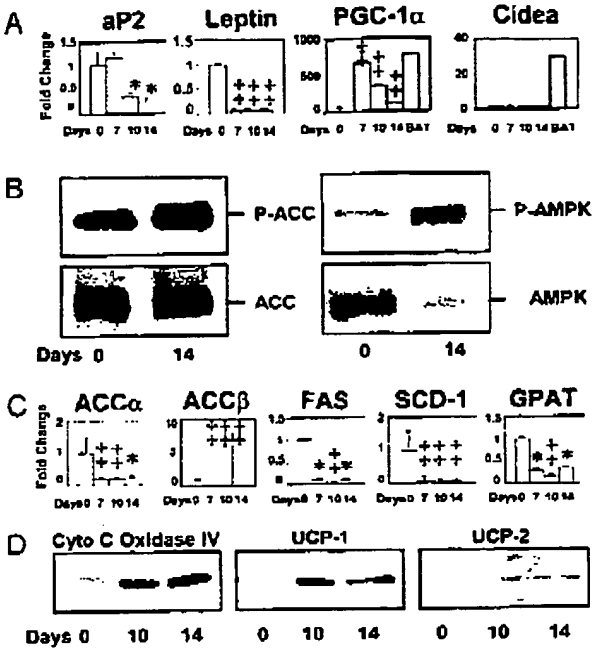


Fig. 3. Effects of adenovirus-induced hyperleptinemia on epididymal white fat tissue. (*A*) Effect of adenovirus-induced hyperleptinemia on the mRNA of genes normally expressed at high levels in mature white adipocytes [adipocyte fatty acid-binding protein 2 (aP_2) and leptin] and on genes normally expressed at high levels only in brown adipocytes (BAT). mRNA is expressed as fold change from 0 time. (0 time: $n = 4$; 7 days: $n = 4$; 10 days: $n = 3$; 14 days: $n = 3$.) •, $P < 0.05$; ‡, $P < 0.01$. (*B*) Effect of adenovirus-induced hyperleptinemia on the total AMPK and Thr-172-phosphorylated α-subunit of AMPK and the total ACC and the Ser-79-phosphorylated ACCα and -β. We assume that it is largely the β isoenzyme, because ACCα mRNA was so strongly down-regulated (see below). Phosphorylation inactivates ACC and activates AMPK. (*C*) Effect of hyperleptinemia on mRNA of key lipogenic enzymes ACCα, ACCβ, fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD-1), and glycerol-3-phosphate acyl transferase (GPAT) expressed as fold change at 7, 10, and 14 days after treatment. •, $P < 0.05$; ‡, $P < 0.01$. (*D*) Immunoblotting showing effects of hyperleptinemia on mitochondrial proteins cytochrome c oxidase IV subunit 5b and UCP-1 and -2.

(shown in Fig. 3*C*) and ATP citrate-lyase (data not shown). Except for ACCβ, which rose ≈8-fold, all were significantly reduced at all points measured after leptin treatment (Fig. 3*C*),

Table 1. Relative changes in transcription factor mRNA content

Transcription factor	Days of hyperleptinemia			
	0 ($n = 4$)	7 ($n = 4$)	10 ($n = 3$)	14 ($n = 3$)
PPARα	1 ± 0.11	0.70 ± 0.16	1.02 ± 0.06	1.18 ± 0.08
PPARγ	1 ± 0.09	0.65 ± 0.09	0.60 ± 0.06	0.87 ± 0.06
PPARδ	1 ± 0.14	1.46 ± 0.31	0.86 ± 0.08	1.33 ± 0.11
LXRα	1 ± 0.20	2.36 ± 0.77*	1.65 ± 0.09*	2.08 ± 0.18*
SREBP-1c	1 ± 0.09	0.45 ± 0.09*	0.91 ± 0.06	1.39 ± 0.26
ChREBP	1 ± 0.31	0.25 ± 0.03*	0.26 ± 0.11*	0.58 ± 0.14*
FOXO1	1 ± 0.10	0.66 ± 0.13*	0.17 ± 0.06*	0.21 ± 0.07*
FOXC2	1 ± 0.18	2.47 ± 0.95*	1.95 ± 0.09*	4.84 ± 0.20*
C/EBPα	1 ± 0.15	1.34 ± 0.35	0.81 ± 0.18	1.65 ± 0.22*

*, $P < 0.05$. LXR, liver X receptor; SREBP, sterol regulatory element-binding protein; ChREBP, carbohydrate-responsive element-binding protein; FOXO1, Forkhead transcription factor FKHR; FOXC2, another Forkhead transcription factor; C/EBP, CCAAT/enhancer-binding protein.

suggesting that reduced biosynthesis of new ACCα, as well as inactivation of ACCβ, may contribute to the fat loss. There was no change in the expression of the oxidative enzymes carnitine-palmitoyl transferase 1 or acyl CoA oxidase (Table 2).

Hyperleptinemia Converts White Adipocytes into Mitochondria-Rich Cells. Conversion of a fat-storing cell into a fat-burning cell would require a substantial up-regulation of mitochondria. The most remarkable ultrastructural finding in postadipocytes was a profusion of filamentous mitochondria (Figs. 2*A* and 4*A*) containing numerous cristae embedded in a highly electron-dense matrix (Fig. 4*B*). The apparent increase in mitochondria cannot be explained entirely by the reduction in cytoplasmic volume resulting from lipid depletion, because starvation-induced volume reduction of adipocytes is not associated with a comparable abundance of mitochondria (20). Moreover, the mitochondria in starvation are pleomorphic and have a less electron-dense matrix (20). The postadipocyte mitochondria differ from those of brown adipocytes, which are much larger and more spherical with cristae that traverse the entire width of the organelle (Fig. 4*C*). There was also an increase in mitochondrial proteins cytochrome *c* oxidase IV and UCP-1 and -2 (Fig. 3*D*) but not in cytochrome *c* (data not shown).

To determine the mechanism of the apparent increase in mitochondria we measured PGC1-α, which regulates mitochondrial biogenesis in brown adipocytes and muscles of normal animals (21–23) but is very low in white adipose tissue. Consistent with the increased mitochondrial abundance, PGC-1α mRNA was strikingly elevated in the postadipocytes, reaching at 7 days after leptinization the levels found in brown adipose tissue (Fig. 3*A*). A progressive decline in PGC-1α mRNA followed as fat disappeared. There were no important changes in the mRNA of various factors involved in PGC-1α activation and actions and in mitochondrial functions other than a decline in the mRNA of nuclear respiratory factor (NRF-1) and mitofusin (Table 2).

Discussion

In the unique type of fat loss induced by hyperleptinemia, the fatty acids appear to be oxidized inside the white adipocytes (2) rather than released and carried to the liver for oxidation to ketones, the classical pathway in other fat-losing conditions (3). In this article, we describe profound morphological and molecular changes in white adipose cells that are consistent with their transformation by hyperleptinemia into fat-burning cells.

Normal-appearing, fat-filled adipocytes were replaced by irregularly shaped, fat-depleted, shrunken cells with extensive infoldings of the cell membrane. These cells could be identified as "former" white adipocytes by tiny residual fat droplets. A thick layer of diffuse basement-membrane-like matrix surrounded these postadipocytes, but the mRNA levels for basement-membrane proteins and for matrix metalloproteinases and their inhibitors provided no clue as to mechanism. Conceivably, the marked decrease in β1 integrin prevented the assembly of basement-membrane components into a condensed extracellular matrix layer (10).

The postadipocytes contained a profusion of mitochondria that differed in size and appearance from the much larger mitochondria of brown adipocytes, in which the cristae traverse the entire organelle. This mitochondrial abundance was accompanied by profound changes in the expression profile of postadipocytes. Most striking of these changes was the increase in PGC-1α mRNA, which rose from the very low levels normally present in white adipose tissue to those of brown adipose tissue (Fig. 3*A*). Because PGC-1α is involved in mitochondrial biogenesis (21–23), its increase may have played a role in the abundance of mitochondria in postadipocytes. Indeed, when PGC-1α is not

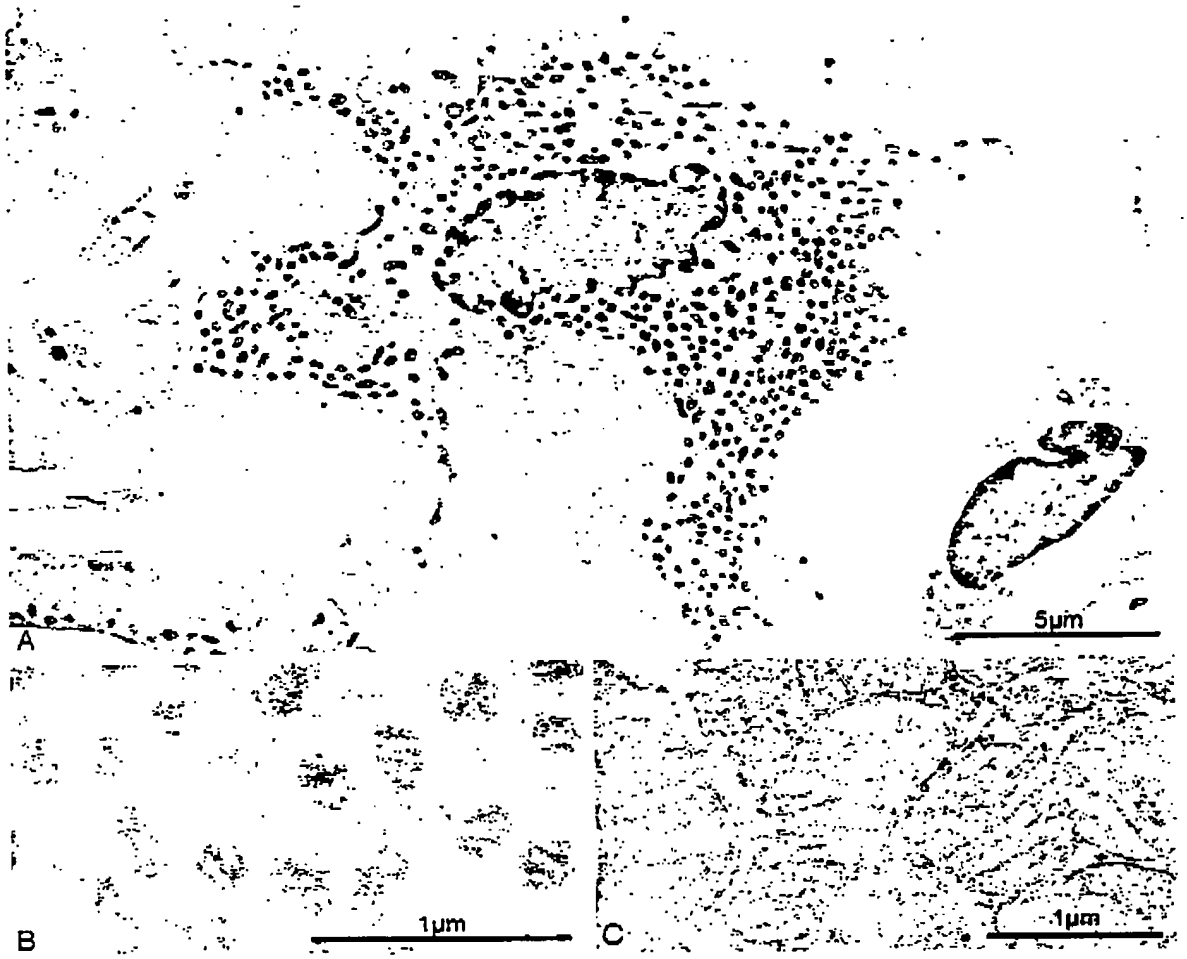


Fig. 4. Mitochondrial ultrastructure in hyperleptinemic white postadipocytes and in brown adipocytes. (*A*) Mitochondrial abundance in the cytoplasm of a postadipocyte. (*B*) Higher magnification of a hyperleptinemia-induced postadipocyte reveals that mitochondrial cristae are embedded in an electron-dense matrix. (*C*) Mitochondria from an untreated brown adipocyte. Although the magnification is lower than that in *B*, mitochondria appear much larger than those of white postadipocytes. In addition, their matrix is less electron-dense than in mitochondria of white postadipocytes, and their cristae traverse the entire width of the organelle.

increased, as in fat cells of adenovirus-leptin-treated PPARα-null mice, the hyperleptinemia fails to induce adipocyte fat loss (12). Because forced expression of PGC-1α in human fat cells enhances their oxidation of fatty acids (24), it is quite likely that its increase was responsible for the loss of fat through "internal combustion."

The intraadipocyte disappearance of fat induced by hyperleptinemia differs sharply from that of starvation and insulin deficiency, in which fatty acids are released from adipocytes and oxidized in the liver. The increased fatty acid oxidation inside white fat tissue can be explained by the changes in the phosphorylation state of key liporegulatory enzymes. The phosphorylation of ACCβ would reduce mitochondrial malonyl CoA (25), thereby increasing fatty acid oxidation, whereas the down-regulation of genes encoding lipogenic enzymes, such as ACCα and other lipogenic enzymes, would reduce their capacity to form new fat. The inactivation of ACCβ by phosphorylation (Fig. 3*B*) would lower malonyl CoA and thus enhance oxidation of the existing fatty acids (16). Finally, the abundance of mitochondria, induced by the high PGC-1α levels, would expand the oxidative machinery required for enhanced oxidation, while increased UCP-1 and -2 protein would dissipate the unneeded energy as heat. Although this combination of events was reminiscent of hyperthyroidism, tri-iodothyronine levels were not increased in the plasma of the leptinized rats.

The metamorphosis of fat-storing white adipocytes into fat-oxidizing cells may have important therapeutic implications for the treatment of obesity. The fat loss induced here was far more rapid and profound than can be induced by caloric restriction. Moreover, it was devoid of the ketonemia, loss of lean body mass, hunger, and other side effects that plague this form of therapy. Control rats pair-fed to the overleptinized animals were clearly unwell, exhibiting ketonemia, reduced physical activity, obvious hunger, and evidence of negative nitrogen balance, whereas the hyperleptinemic rats seemed healthy and normally active or hyperactive, except for disinterest in food. In addition, the fat loss persisted for months after the hyperleptinemia had waned, whereas fat lost through dietary restriction was recovered far more rapidly. This phenomenon could be the result of severe hypoleptinemia induced

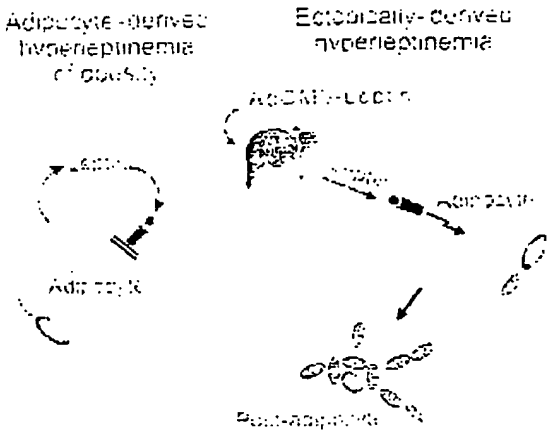


Fig. 5. Comparison of the effects of endogenous (adipocyte-derived; *Left*) and ectopic (liver-derived; *Right*) hyperleptinemia on white adipocyte morphology. (*Left*) As obesity develops, the accumulation of triglycerides is accompanied by progressively increasing hyperleptinemia. The fat-burning action of the hyperleptinemia on the adipocytes is completely blocked, possibly by factor(s) that are coexpressed with leptin. This blockade preserves the physiologic mission of the adipocytes, which is to store fat so as to provide fat fuel during famine. (*Right*) When the hyperleptinemia is ectopically derived, as in normal rats treated with recombinant adenovirus containing the leptin cDNA, there is no blockade of leptin action on adipocytes. Liver-derived hyperleptinemia rapidly transforms adipocytes into mitochondria-rich, fat-burning postadipocytes that are essentially fatless.

by caloric restriction; indeed, it has been demonstrated that caloric restriction plus the maintenance of normoleptinemia with leptin replacement is more effective than caloric restriction alone (26), and it is possible that hyperleptinemia would be even more so.

It is unclear from these results whether the hyperleptinemia acted directly on the adipocytes or by means of a central mechanism. Despite the importance of hypothalamic mediation of leptin actions, there is evidence that, at least at high concentrations, leptin acts directly on white adipocytes. Leptin acts on normal cultured adipocytes *in vitro*, causing effects similar to those observed here (5), and it causes the complete disappearance of fat in denervated fat pads *in vivo* (27), strong evidence for a direct effect, at least in part.

If this interpretation is true, it begs the question as to why the high leptin levels in the interstitial fluid surrounding the adipocytes of rats with diet-induced obesity cannot induce in adipocytes the same changes that are induced by the nonadipocyte-derived hyperleptinemia, even though the interstitial leptin levels must be comparable. We hypothesize that to maintain their vital function of fuel storage and conservation, the adipocytes must mount a powerful defense against their own leptin to prevent wasteful loss of their fat stores, such as we induced here (Fig. 5). If this defense against the leptin that they release could be pharmacologically inactivated or circumvented, a futile cycle would be created in which an increase in intraadipocyte fatty acids would trigger a leptin-induced increase in intraadipocyte fatty acid oxidation. Such an effect would make obesity impossible and might lead to a quick and safe solution of the obesity problem.

We are grateful to A. Widmer and M. Ebrahim Malek for skillful technical assistance and to Christie Fisher and Nadine Dupont for excellent secretarial work. We thank Michael S. Brown, M.D., Cai Li, Ph.D., and Joyce Repa, Ph.D., for critical examination of the manuscript. This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (to R.H.U.) and the Swiss National Science Foundation (to L.O. and R.M.).

1. Chen, G., Koyama, K., Yuan, X., Lee, Y., Zhou, Y. T., O'Doherty, R., Newgard, C. B. & Unger, R. H. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14795–14799.
2. Shimabukuro, M., Koyama, K., Chen, G., Wang, M. Y., Trieu, F., Lee, Y., Newgard, C. B. & Unger, R. H. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 4637–4641.
3. Cahill, G. F., Jr., Aoki, T. T. & Ruderman, N. B. (1973) *Trans. Am. Clin. Climatol. Assn.* **84**, 184–202.
4. Zhou, Y. T., Shimabukuro, M., Koyama, K., Lee, Y., Wang, M. Y., Trieu, F., Newgard, C. B. & Unger, R. H. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 6386–6390.
5. Wang, M. Y., Lee, Y. & Unger, R. H. (1999) *J. Biol. Chem.* **274**, 17541–17544.
6. Liou, W., Geuze, H. J. & Slot, J. W. (1996) *Histochem. Cell Biol.* **106**, 41–58.
7. Bustin, A. (2000) *J. Mol. Endocrinol.* **25**, 169–195.
8. Pierleoni, C., Verdenelli, F., Castellucci, M. & Cinti, S. (1998) *Eur. J. Histochem.* **42**, 183–188.
9. Sternlicht, M. D. & Werb, Z. (2001) *Annu. Rev. Cell Dev. Biol.* **17**, 463–516.
10. Lohikangas, L., Gullberg, D. & Johansson, S. (2001) *Exp. Cell Res.* **265**, 135–144.
11. Zhou, Z., Yon Toh, S., Chen, Z., Guo, K., Ng, C. P., Ponniah, S., Lin, S. C., Hong, W. & Li, P. (2003) *Nat. Genet.* **35**, 49–56.
12. Lee, Y., Yu, X., Gonzales, F., Mangelsdorf, D. J., Wang, M. Y., Richardson, C., Witters, L. A. & Unger, R. H. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 11848–11853.
13. Wang, Y.-X., Lee, C.-H., Tiep, S., Yu, R., Ham, J., Kang, H. & Evans, R. M. (2003) *Cell* **113**, 159–170.
14. Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W. H., III, Arden, K. C. & Accili, D. (2003) *Dev. Cell* **4**, 119–129.
15. Cederberg, A., Grønning, L. M., Ahren, B., Taskén, K., Carlsson, P. & Enerback, S. (2001) *Cell* **106**, 563–573.
16. McGarry, J. D., Mannaerts, G. P. & Foster, D. W. (1977) *J. Clin. Invest.* **60**, 265–270.
17. Hardie, D. G. (2003) *Endocrinology* **144**, 5179–5183.
18. Saha, A. K. & Ruderman, N. B. (2003) *Mol. Cell Biochem.* **253**, 65–70.
19. Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D. & Kahn, B. B. (2002) *Nature* **415**, 339–343.
20. Napolitano, L. & Gagne, H. T. (1963) *Ann. Rec.* **147**, 273–280.
21. Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M. & Kelly, D. P. (2000) *J. Clin. Invest.* **106**, 847–856.
22. Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. & Spiegelman, B. M. (1998) *Cell* **92**, 829–839.
23. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C. & Spiegelman, B. M. (1999) *Cell* **98**, 115–124.
24. Tiraby, S., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D. & Langin, D. (2003) *J. Biol. Chem.* **278**, 33370–33376.
25. Abu-Elheiga, L., Oh, W., Kordari, P. & Wakil, S. J. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 10207–10212.
26. Rosenbaum, M., Murphy, E. M., Heymsfield, S. B., Matthews, D. E. & Leibel, R. L. (2002) *J. Clin. Endocrinol. Metab.* **87**, 2391–2394.
27. Wang, Z.-W., Zhou, Y. T., Lee, Y., Higa, M., Kalra, S. & Unger, R. H. (1999) *Biochem. Biophys. Res. Commun.* **260**, 653–657.

EXHIBIT E

Metabolism and Hormonal Regulation

Stimulation of Gastric Inhibitory Polypeptide Release in *ob/ob* Mice by Oral Administration of Sugars and Their Analogues

PETER R. FLATT,† PIOTR KWASOWSKI AND CLIFFORD J. BAILEY*

*Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, †Department of Biological and Biomedical Sciences, University of Ulster at Coleraine, Co. Londonderry, BT52 1SA and *Biology Division, Department of Pharmaceutical Sciences, Aston University, Birmingham, B4 7ET, United Kingdom*

ABSTRACT The effect of oral administration of sugars and their analogues (glucose, galactose, fructose, mannose, sucrose, *N*-acetylglucosamine, 2-deoxyglucose, 3-O-methylglucose and α-methyl-glucoside) on plasma gastric inhibitory polypeptide (GIP) concentration was examined in 18-h fasted *ob/ob* mice. Administration of sucrose (5.52 mol/kg body wt), or the monosaccharides (11.04 mol/kg body wt) glucose, galactose or fructose, elicited prompt GIP responses that peaked at 30 min. Similar effects were induced by 3-O-methylglucose or α-methyl-glucoside, but the stimulatory action of 2-deoxyglucose was delayed. In contrast to the other sugars, *N*-acetylglucosamine decreased plasma GIP concentration, while mannose exerted no effect. The results suggest that sugars using the Na^+-glucose cotransporter at the luminal brush border stimulate GIP release without the necessity of being metabolized or removed from the cell by the glucose transporter at the basolateral membrane. The ability of fructose to stimulate GIP release in *ob/ob* mice suggests that the Na^+-glucose cotransporter does not represent an exclusive trigger for sugar-induced GIP secretion. *J. Nutr.* 119: 1300–1303, 1989.

INDEXING KEY WORDS:

- *enteroinsular axis*
- *gastric inhibitory polypeptide (GIP)*
- *ob/ob mice*
- *mannose*
- *fructose*
- *sucrose*
- *glucose*
- *galactose*
- *sugar analogues*

Studies in humans and experimental animals have shown that obesity-diabetes syndromes are often associated with increased activity of the enteroinsular axis, as exemplified by an enhanced secretion or insulinotropic action of gastric inhibitory polypeptide (GIP) [1–3]. This abnormality is particularly marked in adult obese hyperglycemic (*ob/ob*) mice that serve as a useful model to investigate the causes and consequences of GIP hypersecretion. Previous studies have indicated that hyperplasia of GIP-secreting K-cells, elevated intestinal GIP content and increased plasma GIP concentration of *ob/ob* mice are determined by the quantity and nutrient composition of the diet [4, 5].

Orally administered glucose, a mixture of amino acids and a fat emulsion each evoked a prominent increase in plasma GIP in adult *ob/ob* mice [6]. Furthermore, neutral and basic amino acids were equipotent, whereas fatty acids produced particularly marked, but variable, GIP responses [7; Flatt, P. R., Kwasowski, P. & Bailey, C. J., unpublished data]. Long-chain fatty acids were more potent stimulators of GIP release than fatty acids that are not esterified into triglycerides following absorption [7]. To further evaluate the specificity and mechanism of nutrient-stimulated GIP release, the present study examined plasma GIP responses of *ob/ob* mice after oral administration of natural sugars and glucose analogues with well-defined intestinal transport characteristics.

MATERIALS AND METHODS

Animals. Groups of obese hyperglycemic [*ob/ob*] mice on the Aston background were used at 12–17 wk of age. The origin and characteristics of these mice have been described elsewhere [8–10]. Briefly, heterozygous C57BL/6J *ob/+* breeding pairs from the Jackson Laboratory, Bar Harbor, ME were obtained in 1957 and outcrossed to two noninbred strains selected for high litter size and increased growth rate. Heterozygous breeding pairs from this stock were transferred to the University of Aston in 1966, where they were used to establish a closed noninbred colony. The severity of the diabetes in the Aston stock is intermediate between that of C57BL/6J and C57BL/KsJ *ob/ob* mice [9, 10].

0022-3166/89 $3.00 Received 21 November 1988. Accepted 10 April 1989.

Mice were housed in an air-conditioned room at 22 ± 2°C with a 12-h light/dark cycle. A standard pelleted nonpurified diet (mouse breeding diet, Heygate & Sons, Northampton, England) and tap water were supplied ad libitum. The diet consisted of 2.5% fat, 17.6% protein and 46.8% carbohydrate (digestible energy 15.3 MJ/kg diet) with added fiber, vitamins and minerals as described elsewhere (11).

Experimental procedure. Sugars (Sigma Chemical, Poole, England) were administered intragastrically to conscious 18-h fasted *ob/ob* mice. The sugars (all D-stereoisomers) tested were glucose, galactose, fructose, mannose, sucrose, *N*-acetylglucosamine, 2-deoxyglucose, 3-O-methylglucose and α-methyl-glucoside. All sugars except sucrose were administered at a dose of 11.04 mmol/(8 ml · kg body wt) corresponding to a dose of 2 g glucose/kg body wt that is commonly employed in glucose tolerance tests (6, 12). Sucrose, which yields equimolar amounts of glucose and fructose on hydrolysis, was given at half of this dose (5.52 mmol/(8 ml · kg body wt)). Blood samples (60 μl) were taken from the tail tip of the mice immediately before and at 30, 60 and 120 min after administration of the sugars.

Analyses. Plasma immunoreactive GIP was measured by double-antibody radioimmunoassay (13) using donkey anti-rabbit gamma globulin antiserum to separate bound and free antigen. Immunoadsorbed GIP-free plasma was used to minimize nonspecific interference, and parallelism was demonstrated between the standard curve and serially diluted *ob/ob* mouse plasma. Natural porcine GIP was used to prepare [^{125}I]GIP and as a standard. The GIP antiserum (RIC34/III), raised in rabbit against a porcine GIP-glutaraldehyde-ovalbumin conjugate, exhibits negligible cross-reactivity with other enteropancreatic hormones.

Statistics. Data were assessed a priori by means of two-way (sugar × time) analysis of variance. Examination of the data in Figure 1 indicated heteroscedasity (Hartley's F_{max} = 105). These data were therefore analyzed following $\log_{10}$ transformation (Hartley's F_{max} = 49, indicating homoscedasity). Differences between individual data points were assessed by one-way analysis of variance, followed by Duncan's multiple range analysis. Differences were considered to be significant if $P < 0.05$.

RESULTS

Plasma GIP responses of *ob/ob* mice to orally administered sugars are shown in Figure 1. Of the sugars tested, only mannose lacked a significant effect on plasma GIP concentration. Administration of sucrose, glucose, galactose or fructose elicited prompt GIP re-

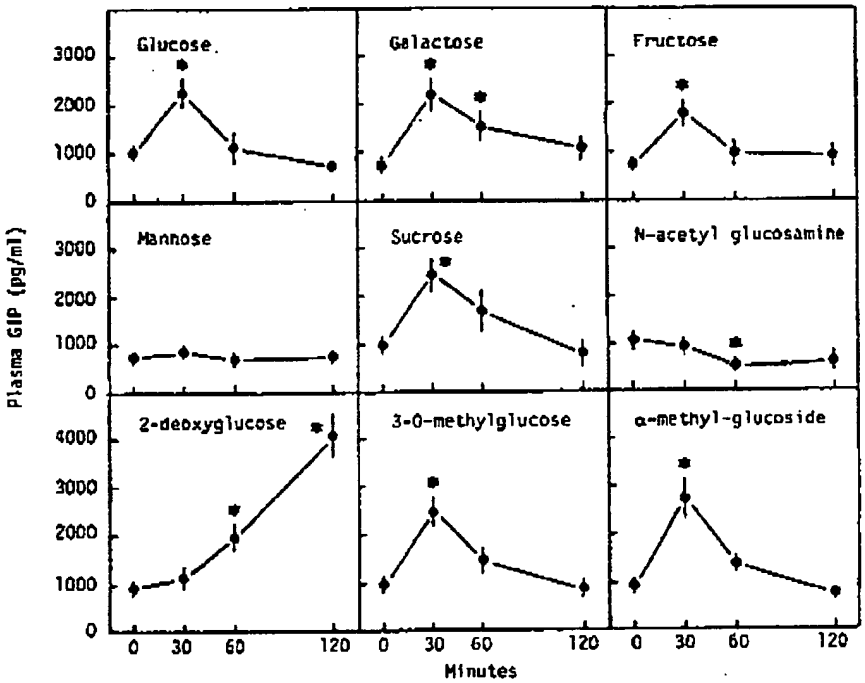


FIGURE 1 Plasma gastric inhibitory polypeptide (GIP) responses to sugars in 18-h fasted *ob/ob* mice. Values are means ± SEM of groups of five mice. * $P < 0.05$ compared with time 0. ANOVA confirmed that plasma GIP concentration was significantly affected by time and by administration of different sugars ($P < 0.0001$). There was also a significant interaction between these two variables ($P < 0.0001$).

TABLE 1

Effects of sugars on plasma gastric inhibitory polypeptide (GIP) concentration in 18-h fasted ob/ob mice[1]

Sugar	Incremental GIP response (0–30 min)	Integrated GIP response (0–120 min)
	pg/ml	*pg/(ml·h)*
Glucose	1215 ± 384[ab]	313 ± 456[ab]
Galactose	1467 ± 366[ab]	1491 ± 175[b]
Fructose	864 ± 353	861 ± 316[ab]
Mannose	79 ± 341	−144 ± 393[a]
Sucrose	1412 ± 315[ab]	893 ± 470[ab]
N-Acetylglucosamine	−148 ± 163	−994 ± 233[a]
2-Deoxyglucose	113 ± 243	2367 ± 623[b]
3-O-Methylglucose	1496 ± 440[ab]	1030 ± 520[ab]
α-Methyl-glucoside	1806 ± 515[ab]	1112 ± 366[ab]

[1]Values are means ± SEM of groups of five mice. Superscript letters indicate $P < 0.05$ compared with [a]2-deoxyglucose and [b]*N*-acetylglucosamine, as assessed by Duncan's multiple range analysis. The incremental GIP responses and the integrated GIP responses to glucose, galactose, fructose, sucrose, 3-O-methylglucose and α-methyl-glucoside were not significantly different, as assessed by Duncan's multiple range analysis.

sponses that peaked at 30 min. Similarly rapid effects were observed in response to the analogues 3-O-methylglucose or α-methyl-glucoside. However, the stimulatory action of 2-deoxyglucose was considerably delayed, with GIP concentration continuing to increase at 120 min. In contrast to the other sugars, *N*-acetylglucosamine decreased plasma GIP concentration by 60 min.

Table 1 summarizes the incremental GIP response between 0 and 30 min, as well as the overall integrated response. The latter was calculated as: pg/(ml · h) = [GIP at (30 + 60 + 120 min) − (3 · basal GIP)]/2. No significant differences were noted in the incremental response of those sugars that exerted prompt stimulatory effects on GIP release, namely, sucrose, glucose, galactose, fructose, 3-O-methylglucose and α-methyl-glucoside. The overall integrated GIP response to these sugars was also similar. Compared with the other sugars, the overall integrated GIP response to 2-deoxyglucose was significantly enhanced (except compared with galactose), and the response to *N*-acetylglucosamine was significantly reduced (except compared with mannose).

DISCUSSION

Hyperinsulinemia is an early pathogenic feature in obese hyperglycemic (*ob/ob*) mice which makes an important contribution to the severity of insulin resistance and glucose intolerance (10). Short-term feeding of *ob/ob* mice with isoenergetic diets of different nutrient composition has shown that carbohydrate represents the major stimulus to the hyperinsulinemia (11, 14). Since intraperitoneal injection of glucose fails to elicit an insulin response in nonfasted *ob/ob* mice (12), attention has focused on the involvement of the overactive enteroinsular axis and hypersecretion of GIP in the hyperinsulinemia of these mice (1). The present study has shown that a range of naturally occurring sugars, including glucose, galactose, fructose and sucrose, elicit rapid and prominent GIP responses in *ob/ob* mice. This observation, together with the earlier demonstration (6) that GIP provides a physiological stimulus to insulin secretion in *ob/ob* mice, offers a mechanistic link between the ingestion of carbohydrate and the hyperinsulinemia.

The present observation that sugars that serve as a substrate for the Na^+-glucose cotransporter in the luminal brush border (15, 16) elicit GIP response is consistent with results obtained by single time-point sampling of the hepatic portal vein of anesthetized rats (17). Thus, glucose, galactose, α-methyl-glucoside and 3-O-methylglucose were equally effective in raising the plasma GIP concentration of *ob/ob* mice. Although these sugars utilize the same transport carrier at the brush border, α-methyl-glucoside is not a substrate for the glucose transporter at the basolateral membrane, and 3-O-methylglucose is not metabolized within the intestinal cells (16). This indicates that the GIP-releasing action of glucose and galactose requires neither their metabolism nor transport out of the cell. Nevertheless, the observation that fructose increased plasma GIP in *ob/ob* mice raises doubt as to whether the Na^+-glucose cotransporter represents the sole trigger to GIP secretion. Although not confirmed in mice, fructose absorption is generally considered to involve a brush border transporter independent of both Na^+ and the glucose transport system (15, 16).

The GIP-releasing action of fructose in *ob/ob* mice contrasts with the reported lack of effect of this sugar on circulating GIP concentration in normal human subjects and rats (17, 18). This lack of effect in the rat may reflect the use of a single blood sample to assess GIP release 30 min after luminal perfusion of the sugar (17). The divergence from humans represents either a pathological feature associated with obesity-diabetes or a peculiarity to mice. In this respect it is notable that sucrose, which is hydrolyzed to glucose and fructose by brush border enzymes, elicited a GIP response comparable to twice the molar dose of glucose or fructose alone. Since the effect of glucose on GIP release is dose-dependent in all species studied (18–20), a smaller GIP response to sucrose would be predicted if fructose were not stimulatory. Consistent with this observation, an equimolar concentration of glucose and sucrose elicited comparable GIP responses in the rat (17). Moreover, it is of interest that incorporation of fructose, instead of glucose, into the diet of *db/db* mice did not ameliorate the diabetes-obesity syndrome (21).

Absorption of mannose, 2-deoxyglucose and *N*-acetylglucosamine involves neither the glucose nor fructose luminal transport system (15, 16). Mannose did not elicit a GIP response, whereas 2-deoxyglucose pro-

duced a delayed, but substantial, elevation of plasma GIP concentration. The latter effect suggests an action that is independent of intestinal transport, such as activation of the sympathetic nervous system [22] and the release of somatostatin [23]. The mechanism underlying the inhibitory effect of *N*-acetylglucosamine on circulating GIP concentrations is also unclear. Although this sugar stimulates insulin release in the rat [24], suppression of plasma GIP does not involve insulin-feedback inhibition of intestinal K-cells, which is defective in *ob/ob* mice [6].

ACKNOWLEDGMENTS

The authors wish to thank R. J. Howland and V. Marks for constructive comments.

LITERATURE CITED

1. Bailey, C. J. & Flatt, P. R. (1988) The enteroinsular axis in models of hyperinsulinaemic and hypoinsulinaemic diabetes. In: *Frontiers in Diabetes Research: Lessons from Animals Diabetes*, Vol. 2 (Shafrir, E. & Renold, A. E., eds.), pp. 217–224, John Libbey, London.
2. Morgan, L. M., Flatt, P. R. & Marks, V. (1988) Nutrient regulation of the enteroinsular axis and insulin secretion. *Nutr. Res. Rev.* 1: 79–97.
3. Krarup, T. (1988) Immunoreactive gastric inhibitory polypeptide. *Endocr. Rev.* 9: 122–134.
4. Flatt, P. R., Bailey, C. J., Kwasowski, P., Swanston-Flatt, S. K. & Marks, V. (1985) Glucoregulatory effects of cafeteria feeding and diet restriction in genetically obese hyperglycaemic (*ob/ob*) mice. *Nutr. Rep. Int.* 32: 847–854.
5. Bailey, C. J., Flatt, P. R., Kwasowski, P., Powell, C. J. & Marks, V. (1986) Immunoreactive gastric inhibitory polypeptide and K-cell hyperplasia in obese hyperglycaemic (*ob/ob*) mice fed high fat and high carbohydrate cafeteria diets. *Acta Endocrinol.* (Copenhagen) 112: 224–229.
6. Flatt, P. R., Bailey, C. J., Kwasowski, P., Page, T. & Marks, V. (1984) Plasma immunoreactive gastric inhibitory polypeptide in obese hyperglycaemic (*ob/ob*) mice. *J. Endocrinol.* 101: 249–256.
7. Kwasowski, P., Flatt, P. R., Bailey, C. J. & Marks, V. (1985) Effects of fatty acid chain length and saturation on gastric inhibitory polypeptide release in obese hyperglycaemic (*ob/ob*) mice. *Biosci. Rep.* 5: 701–705.
8. Flatt, P. R. & Bailey, C. J. (1981) Abnormal plasma glucose and insulin responses in heterozygous lean (*ob/+*) mice. *Diabetologia* 20: 573–577.
9. Bailey, C. J., Flatt, P. R. & Atkins, T. W. (1982) Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston *ob/ob* mice. *Int. J. Obes.* 6: 11–21.
10. Bailey, C. J. & Flatt, P. R. (1986) Animal models of diabetes. In: *Recent Advances in Diabetes*, Vol. 2 (Nattrass, M., ed.), pp. 71–89, Churchill Livingstone, Edinburgh.
11. Flatt, P. R. & Bailey, C. J. (1982) Role of dietary factors in the hyperinsulinemia of genetically obese hyperglycemic (*ob/ob*) mice. *J. Nutr.* 112: 2212–2216.
12. Flatt, P. R. & Bailey, C. J. (1981) Development of glucose intolerance and impaired plasma insulin response to glucose in obese hyperglycaemic (*ob/ob*) mice. *Horm. Metabol. Res.* 13: 556–560.
13. Morgan, L. M., Morris, B. & Marks, V. (1978) Radioimmunoassay of gastric inhibitory polypeptide. *Ann. Clin. Biochem.* 15: 172–177.
14. Flatt, P. R. & Bailey, C. J. (1984) Dietary components and plasma insulin responses to fasting and refeeding in genetically obese hyperglycaemic (*ob/ob*) mice. *Br. J. Nutr.* 51: 403–413.
15. Kimmich, G. A. (1981) Intestinal absorption of sugar. In: *Physiology of the Gastrointestinal Tract*, 1st ed. (Johnson, L. R., ed.), pp. 1035–1061, Raven Press, New York.
16. Hopfer, U. (1987) Membrane transport mechanisms for hexoses and amino acids in the small intestine. In: *Physiology of the Gastrointestinal Tract*, 2nd ed. (Johnson, L. R., ed.), pp. 1499–1526, Raven Press, New York.
17. Sykes, S., Morgan, L. M., English, J. & Marks, V. (1980) Evidence for preferential stimulation of gastric inhibitory polypeptide secretion in the rat by actively transported carbohydrates and their analogues. *J. Endocrinol.* 85: 201–207.
18. Morgan, L. M. (1979) Immunoassayable gastric inhibitory polypeptide: Investigations into its role in carbohydrate metabolism. *Ann. Clin. Biochem.* 16: 6–14.
19. Pederson, R. A., Schubert, H. E. & Brown, J. C. (1975) Gastric inhibitory polypeptide. Its physiological release and insulinotropic action in the dog. *Diabetes* 24: 1050–1056.
20. Martin, E. W., Sirinek, K. R., Crockett, S. E., O'Dorisio, T. M., Mazzaferri, E. L., Thomford, N. R. & Cataland, S. (1975) Release of gastric inhibitory polypeptide: Comparison of hyperosmolar carbohydrate solutions as stimuli. *Surg. Forum* 26: 381–382.
21. Leiter, E. H., Coleman, D. L., Ingram, D. K. & Reynolds, M. A. (1983) Influence of dietary carbohydrate on the induction of diabetes in CS7BL/Ks-*db/db* diabetes mice. *J. Nutr.* 113: 184–195.
22. Fisher, D. A. & Brown, M. R. (1980) Somatostatin analogue: Plasma catecholamine suppression mediated by the central nervous system. *Endocrinology* 107: 714–718.
23. Bodan, C., Master, R. W., Sattler, M. A., Martin, J. S., Tansy, M. F. & Owen, O. E. (1982) Adrenergic control of somatostatin release. *Endocrinology* 111: 1166–1172.
24. Ashcroft, S. J. H. & Crossley, J. R. (1975) The effect of glucose, *N*-acetylglucosamine, glyceraldehyde and other sugars on insulin release in vivo. *Diabetologia* 11: 279–284.

EXHIBIT F

Augmented Release of Gastric Inhibitory Polypeptide into the Portal Vein in Response to Intraduodenal Glucose and Amino Acids in Anesthetized Rats Treated with Methylprednisolone or Alloxan

T. B. SCHULZ, R. JORDE, P. G. BURHOL & O CHRISTENSEN
Laboratory of Gastroenterology, Dept. of Medicine,
University of Tromsø, Tromso, Norway

Schulz TB, Jorde R, Burhol PG, Christensen O. Augmented release of gastric inhibitory polypeptide into the portal vein in response to intraduodenal glucose and amino acids in anesthetized rats treated with methylprednisolone or alloxan. Scand J Gastroenterol 1982; 6: 357–362.

Thirty rats were treated with methylprednisolone, 30 rats were treated with alloxan, and 30 control rats were treated with saline alone. The levels of fasting serum insulin and blood glucose and plasma GIP before and after duodenal instillation of glucose or amino acids were measured using an acute rat preparation that enabled multiple blood samplings from the portal vein. Treatment of the rats with methylprednisolone was followed by increased fasting levels of serum insulin, blood glucose, and plasma GIP and by an augmented GIP release in response to duodenal glucose and amino acids as compared with normal controls. Similarly, treatment with alloxan was followed by decreased fasting levels of serum insulin, by increased fasting levels of blood glucose and plasma GIP, and by an increased GIP release in response to duodenal glucose and amino acids. The augmented GIP release in response to duodenal instillation of glucose and amino acids both in methylprednisolone-treated rats and in alloxan-treated rats may be explained by an increased absorption of these nutrients owing to an increased Na^+ K^+ ATPase activity in the intestinal mucosa of corticosteroid- and alloxan-treated rats. The elevated fasting GIP levels, on the other hand, are difficult to explain.

Key words: Alloxan; amino acid infusion; diabetes, experimental; gastric inhibitory polypeptide (GIP); glucose, duodenal; methylprednisolone; rats

T. B. Schulz, M.D., Dept. of Medicine, Askim sykehus, 1800 Askim, Norway

In alloxan-treated rats, in untreated juvenile diabetics, and in hyperinsulinemic obese subjects, an elevated fasting plasma GIP level and an augmented GIP release in response to glucose have been reported (1–10). The cause of the elevated GIP levels is unknown, but disturbance of a postulated negative feedback effect by insulin on GIP release has been suggested.

The release of GIP after glucose and amino acid stimulation seems to depend more on an active absorption than on their presence within the intestinal lumen (3, 11–14). The active absorption is an Na^+-dependent process coupled to the Na^+ K^+ ATPase of the intestinal cells (15, 16). Treatment of rats with alloxan is known to induce insulin deficiency and diabetes and to increase glucose and amino acid absorption (17–19), which probably is effected by an increased mucosal Na^+ K^+ ATPase activity. Even though corticosteroid-treated rats have elevated levels of serum insulin, they also have raised blood glucose levels and increased glucose and amino acid absorption together with an increased mucosal Na^+ K^+ ATPase activity (20–24).

Provided that the effect of an increased absorption is more important than negative feedback control by insulin, one would expect increased GIP release in response to glucose and amino acids both in alloxan hypoinsulinemic rats and in corticosteroid hyperinsulinemic rats. The present study was therefore undertaken to study this hypothesis.

MATERIALS AND METHODS

Experimental design. An acute rat preparation as previously described (13), using male Wistar rats, 300–320 g, of uniform breed kept on a standard diet (Standard rat food, Felleskjøpet, Trondheim, Norway) for at least 3 weeks before the experiments, was used.

Anesthesia was achieved by intraperitoneal injection of sodium pentobarbital (Nembutal®; Abbott Laboratories, Chicago, Ill.), 50 mg/kg body weight. The abdomen was opened, and the small bowel was ligated close to the pylorus and 30 cm proximal to the ileocecal junction and then displaced to the left to give easy access to the portal vein. Urine was obtained by bladder puncture. The body temperature was kept constant at 38°C by using an electric heating pad throughout the experiments.

After a 20-min recovery period the experiments were started. The glucose, amino acid, and saline solutions were instilled as a bolus of 20 ml/kg body weight into the proximal part of the duodenum. The pHs of the solutions were adjusted with HCl to pH 5.2, and their osmolalities to iso-osmolality with NaCl.

Treatment with methylprednisolone. The procedure of Charney et al. (20) for induction of Na^+ K^+ ATPase in the small-intestinal mucosa of rats was used. Thirty rats were given three intramuscular injections with methylprednisolone (Depo-Medrol®; Upjohn International Inc., Puurs, Belgium), 30 mg/kg body weight with 24-h intervals. After the final injection, the animals were fasted for 20 h with water allowed freely, before the experiments were started.

Treatment with alloxan. Thirty rats were given one intramuscular injection with alloxan (Sigma Chemical Co., St. Louis, Mo.), 200 mg/kg body weight after a 20-h fast. The animals were then allowed to eat freely for 24 h. Before the experiments were started the animals were fasted for another 20 h, with water allowed freely.

Treatment with saline. Thirty control rats were given intramuscular injections with saline, 1.0 ml/kg body weight, following the same procedure as described under treatment with methylprednisolone.

Duodenal instillation of glucose. In 10 methylprednisolone-treated rats, in 10 alloxan-treated rats, and in 10 control rats portal vein blood for estimation of plasma GIP, serum insulin, blood glucose, and packed cell volume was drawn 1 min before instillation of 0.695 mmol glucose per kilogram body weight, and, in addition, portal vein blood for determination of plasma GIP was drawn 5 and 30 min after the duodenal glucose instillation.

Duodenal instillation of amino acids. The experimental procedure was as described above, except that an amino acid solution (Kabi AB, Stockholm, Sweden), 1.379 mmol/kg body weight (Table I), was instilled into the duodenum instead of glucose.

Duodenal instillation of saline. The experimental procedure was as described above, except that saline alone was instilled into the duodenum.

Table 1. The composition of the amino acid solution used in the experiments

Amino acids	Mmol/l
L-Alanine	4.2
L-Arginine	2.1
L-Aspartic acid	3.9
L-Cystine	0.7
L-Cysteine	1.3
L-Glutamic acid	7.7
Glycine	3.5
L-Histidine	1.7
L-Isoleucine	3.7
L-Leucine	5.1
L-Lycine	3.0
L-Methionine	1.6
L-Phenylalanine	4.2
L-Proline	8.8
L-Serine	8.9
L-Threonine	3.2
L-Tryptophane	0.6
L-Tyrosine	0.4
L-Valine	4.6
Total	69.0

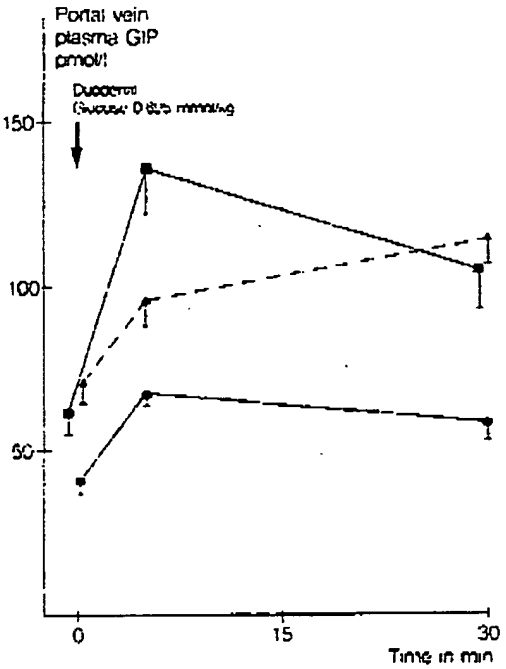


Fig. 1. Portal vein plasma GIP before and after duodenal instillation of glucose, 0.695 mmol/kg body weight, in rats treated with (■—■) methylprednisolone, (▲—▲) alloxan, or (●—●) saline. Each point represents the mean of one examination in each of 10 rats, and the vertical bars the standard errors of the means.

levels (± S.E.M.) in the methylprednisolone-, alloxan-, and saline-treated rats were 61.4 ± 7.2 pmol/l, 70.9 ± 5.9 pmol/l, and 40.3 ± 3.3 pmol/l, respectively. Mean portal vein plasma GIP rose significantly at 5 min and stayed significantly elevated 30 min after duodenal instillation of 0.695 mmol glucose per kilogram body weight in the three individual groups of rats (Fig. 1).

Mean fasting portal vein plasma GIP and mean portal vein plasma GIP 5 and 30 min after duodenal glucose were significantly higher in the methylprednisolone- and in the alloxan-treated rats than in the saline-treated control rats.

Portal vein plasma GIP before and after duodenal instillation of amino acids

The mean fasting portal vein plasma GIP levels (± S.E.M.) in the methylprednisolone-, alloxan-, and saline-treated rats were 55.2 ± 3.2 pmol/l, 84.5 ± 9.3 pmol/l, and 39.4 ± 1.8 pmol/l, respectively. Mean portal vein plasma GIP rose significantly at 5 min and stayed significantly elevated 30 min after duodenal instillation of 1.379 mmol amino acids per kilogram body weight in the three individual groups of rats (Fig. 2).

Mean fasting portal vein plasma GIP and mean portal vein plasma GIP levels 5 and 30 min after duodenal amino acids were significantly higher in the methylprednisolone-treated and in the alloxan-treated rats than in the saline-treated control rats.

Portal vein plasma GIP before and after duodenal instillation of saline

The mean portal vein plasma GIP level (± S.E.M.) rose non-significantly from 59.4 ± 3.9 pmol/l before to 60.6 ± 5.1 pmol/l 30 min after duodenal saline in 10 methylprednisolone-treated

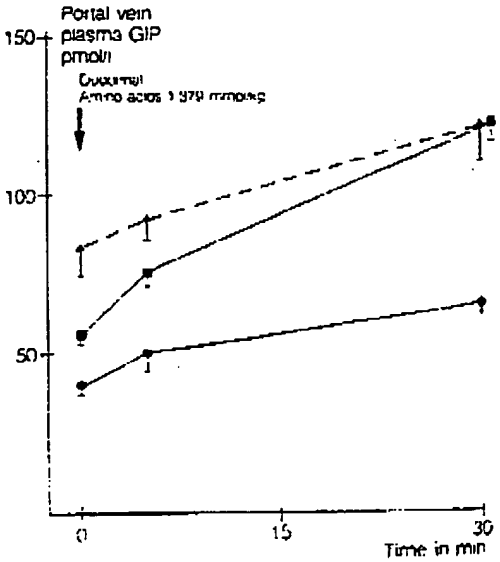


Fig. 2. Portal vein plasma GIP before and after duodenal instillation of an amino acid solution, 1.379 mmol/kg body weight, in rats treated with (■—■) methylprednisolone, (▲—▲) alloxan, or (●—●) saline. Each point represents the mean of one examination in each of 10 rats, and the vertical bars the standard errors of the means.

It has, however, been suggested that the GIP cells in these insulin-resistant patients may have a decreased sensitivity to insulin (3). Our findings of elevated fasting plasma GIP in the methylprednisolone-treated rats with elevated fasting serum insulin may be explained by the same effect, since it has been demonstrated that administration of corticosteroids to rats leads to decreased binding of insulin to rat adipocytes and hepatocytes (7, 25). Whether steroids exert a similar effect on the GIP-producing cells is, however, unknown. The reason for the elevated fasting plasma GIP level when fasting serum insulin also is elevated must therefore still be considered unknown.

In conclusion, treatment of rats with methylprednisolone was followed by increased fasting levels of serum insulin, blood glucose, and plasma GIP and also by an augmented GIP release in response to duodenal glucose and amino acids. Similarly, treatment of rats with alloxan was followed by decreased fasting levels of serum insulin, by increased fasting levels of blood glucose and plasma GIP, and by an increased release of GIP in response to duodenal glucose and amino acids. The elevated fasting GIP levels are difficult to explain, whereas the augmented GIP release may possibly be ascribed to an increased absorption of glucose and amino acids in these animals subsequent to a raised Na^+ K^+ ATPase activity known to occur in the intestinal mucosa of alloxan- or corticosteroid-treated rats.

ACKNOWLEDGEMENTS

We appreciate the excellent technical assistance of Ingerid Arbo, Aase Bendiksen, Ingebjørg Einarsdottir, and Lise Bjørnson Schulz. This study was supported by Helga Sembs Fond, Norsk Råd til Bekjempelse av Fordøyelsessykdommer, Nordisk Insulinfond, Norsk Forening til Kreftens Bekjempelse, and the Norwegian Research Council for Science and the Humanities.

REFERENCES

1. Brown JC, Dryburgh JR, Ross SA, Dupré J. Recent Prog Horm Res 1975, 31, 487–532
2. Coxe JS, O'Dorisio TM, Cataland S, Crockett SE. J Clin Endocr Metab 1981, 52, 1002–1005
3. Creutzfeldt W, Ebert R. Excerpta Medica Int Congr Ser 1977, 413, 63–75
4. Crockett SE, Mazzaferri EL, Cataland S. Diabetes 1976, 25, 931–935
5. Ebert R, Frerichs H, Creutzfeldt W. Diabetologia 1976, 12, 388
6. O'Dorisio TM, Cataland D, Lee GH, Reed DK, Mazzaferri EL. Excerpta Medica Int Congr Ser 1979, 481, 175–176
7. Ross SA, Brown JC, Dupré J. Diabetes 1977, 26, 525–529
8. Ross SA, Dupré J. Diabetes 1978, 27, 327–333
9. Talaulicar M, Ebert R, Willms B, Creutzfeldt W. Clin Endocr 1981, 14, 175–180
10. Willms B, Ebert R, Creutzfeldt W. Diabetologia 1978, 14, 379–387
11. Creutzfeldt W, Ebert R, Arnold R, Frerichs H, Brown JC. Diabetologia 1976, 12, 279–286
12. Morgan LM. Ann Clin Biochem 1979, 16, 6–14
13. Schulz TB, Burhol PG, Jorde R, Waldum HL. Scand J Gastroenterol 1981, 16, 1061–1065
14. Schulz TB, Jorde R, Burhol PG. Scand J Gastroenterol 1982, in press
15. Schultz SG, Curran PF. Physiol Rev 1970, 50, 637–718
16. Silk DBA. Proc Nutr Soc 1980, 39, 61–70
17. Crane RK. Biochem Biophys Res Commun 1961, 4, 436–440
18. Fromm D, Field M, Silen W. Am J Physiol 1969, 217, 53–57
19. Olson W, Rosenberg IH. J Clin Invest 1970, 49, 96–105
20. Charney AN, Kinsey MD, Meyers L, Giannella AR, Gots RE. J Clin Invest 1975, 56, 653–660
21. Gnanaprakasam MS, Srivastava LM. Biochem Exp Biol 1978, 14, 257–262
22. Mueller F, Beyreiss K, Dettmer D, Hartenstein H. Acta Biol Med Ger 1967, 19, 673–681
23. Rachmilewitz D, Fogel R, Karmeli F. Gut 1978, 19, 759–764
24. Wilbrandt W, Lengyel L. Biochem Z 1933, 267, 204–210
25. Heding LG. Diabetologia 1972, 8, 260–266
26. Banerjee S, Varma SD. Proc Soc Exp Biol Med 1966, 123, 212–213
27. Luppa D, Hønicke S, Reissig D, Mueller F. Acta Biol Med Ger 1978, 37, 39–47
28. Flores P, Schedl HP. Am J Physiol 1968, 214, 725–729
29. Laszt L, Vogel B. Nature 1946, 157, 551–552
30. Sols A, Vidal S, Larraldc J. Nature 1948, 161, 932–933
31. Andersen DK, Elahi D, Brown JC, Tobin JD, Andres R. J Clin Invest 1978, 62, 152–161
32. Reynolds C, Tronsgard N, Gibbons E, Blix PM, Rubenstein AH. J Clin Endocr Metab 1979, 49, 255–261
33. Creutzfeldt W, Talaulicar M, Ebert R, Willms B. Diabetes 1980, 29, 140–145

Received 7 July 1981
Accepted 15 November 1981

EXHIBIT G

Gastric Inhibitory Polypeptide (GIP) Stimulated by Fat Ingestion in Man

JAMES M. FALKO, SAMUEL E. CROCKETT, SAMUEL CATALAND, AND ERNEST L. MAZZAFERRI

Division of Endocrinology and Metabolism, Department of Medicine, Ohio State University, Columbus, Ohio 43210

ABSTRACT. Ten normal volunteers ingested emulsified corn oil and the immunoreactive GIP, insulin (IRI) and nonesterified fatty acid (NEFA) responses were measured. Serum GIP levels increased after the ingestion of corn oil in all subjects, rising from a mean fasting level of 272 pg/ml to 856 ± 272 pg/ml ($P < 0.05$) by 30 minutes. The peak mean serum GIP concentration of 1,345 ± 291 pg/ml occurred at 60 minutes; and mean serum GIP levels at 180 minutes remained significantly elevated over fasting values. Serum IRI, glucose and NEFA concentrations did not change during the 180 minutes of study. No changes in serum GIP concentrations occurred when, for control purposes, six volunteers ingested water on another day. We conclude: 1) Fat is a potent stimulus for the release of GIP in normal individuals. 2) Endogenously released GIP is not insulinotropic under the conditions of this study. (*J Clin Endocrinol Metab* 41: 260, 1975)

GASTRIC inhibitory polypeptide (GIP) appears to exert at least two diverse physiologic effects: one, an enterogastrone effect and the other, an insulinotropic effect. Brown (1) suggests that GIP satisfies all the criteria for being the enterogastrone originally described by Kosaka and Lim (2). Additionally, highly purified porcine GIP is insulinotropic in man (3). Since oral glucose is a potent stimulus for the endogenous release of GIP in normal subjects, (4) it has been suggested that this polypeptide may play an important role in mediating the enteroinsular axis (1,3,4). Dupré and associates (3) report that porcine GIP is insulinotropic in man only in the presence of hyperglycemia. However, Brown and colleagues (1) subsequently suggested that GIP stimulates insulin release when administered *without* intravenous glucose.

Serum GIP concentrations increase in normal subjects following a test meal (5) or after the ingestion of glucose (4). Brown (6) has suggested in a preliminary report that Lipomul, a suspension of corn oil, also stimulates GIP secretion to levels slightly less than those observed following a standard meal (5). Serum insulin levels were not mentioned in Brown's preliminary report (6) of a GIP response to Lipomul; although, ordinarily, the ingestion of fat does not elicit any significant insulin secretion in man (7). We have studied the immunoreactive GIP and insulin responses to corn oil in normal subjects and find that, while this is a potent stimulus to the release of GIP, insulin secretion does not occur. Thus, endogenously released GIP is not insulinotropic in normal subjects under the conditions of this study.

Materials and Methods

Ten healthy, ambulatory, normal volunteers (7 men and 3 women) with a mean age of 25, and ranging from 19 to 31 yr, were studied. No subject was taking any medicine, and all were within 10% of their ideal body weight. After an overnight fast, each volunteer was given 67 g of corn oil in the form of Lipomul* (100 ml), which

Received January 13, 1975.

Address inquiries to: Ernest L. Mazzaferri, M.D., Division of Endocrinology and Metabolism, Room N-1111, University Hospital, 410 W. 10th Avenue, Columbus, Ohio 42310.

*100 ml of Lipomul contains 67 g of corn oil consisting of linoleic acid 34–62%, oleic 19–49%, palmitic acid 8–12%, stearic acid 2.5–4.5%, myristic acid 0.1–1.7%, and hexadecenoic acid 0.2–1.6%. There is no glucose or carbohydrate in the vehicle.

was ingested within 2 minutes. Venous blood samples were drawn at fasting, 15, 30, 60, 90, 120, and 180 min. For control purposes, on a different morning, similar studies were done on six of the volunteers after the ingestion of 100 ml of water.

Blood samples were immediately centrifuged at room temperature and the serum frozen and stored at −20 C. Serum glucose and nonesterified fatty acids (NEFA) were measured immediately after separating the serum. Immunoreactive GIP and insulin (IRI) levels were determined on specimens which had been frozen and stored for no longer than 2 weeks.

Serum glucose was measured in duplicate on each sample by the glucose oxidase method using a Beckman glucose analyzer. Duplicate glucose determinations agreed within ±3 mg% and the average values are reported. NEFA was determined by a modification of Dole's method (8). Serum IRI was measured by means of immunosorbent radioimmunoassay in which the antibody is coupled covalently to cross-linked dextran, Sephadex (9).

Immunoreactive GIP was determined by a modification of the method of Kuzio and associates (5). Highly purified (Stage 4) porcine GIP was labelled with ^{125}I according to the technique of Hunter and Greenwood (10). Guinea pig antiserum to porcine GIP has no demonstrable cross reactivity with motilin, natural porcine secretin, synthetic glucagon, synthetic human gastrin, highly purified cholecystokinin (CCK) and vasoactive intestinal polypeptide (VIP) (4,5). The lower sensitivity of the assay in our laboratory is ordinarily between 25 and 50 pg, allowing the detection of 125 to 250 pg/ml of GIP in serum. Serum aliquots of 200 μl were assayed in duplicate. Bound and free hormone were separated by the double antibody technique. All GIP values reported after fat ingestion in this study were determined in a single assay to avoid between assay variability. In this assay the lower limit of sensitivity was 250 pg/ml. Values of less than 250 pg/ml were arbitrarily considered as 250 pg for statistical analysis. Within assay variance was 8.0%. For GIP values previously reported after glucose ingestion the within assay variance was 9.5%, between assay variance was 17% and standard pooled serum varied ±37.9 pg/ml (SEM).

Results were analyzed by standard statistical methods using the paired t test to compare values among those given a corn oil stimulus. The two-sample *t* test was used to compare results between those individuals previously given glucose and the volunteers in this study who ingested fat. A *P* value of less than 0.05 was considered significant.

Results

Mean serum GIP values in the 10 volunteers who ingested 67 g of corn oil are summarized in Fig. 1. These results are compared to those obtained during the control period when water was ingested. In the control studies summarized in Table 1 most GIP values were at or below the lower limits of sensitivity of the assay. By contrast, a significant increase in serum GIP concentrations occurred in all 10 individuals studied following the ingestion of corn oil. Although mean fasting GIP concentrations were 250 pg/ml or less in 9 of 10 study subjects, one individual had a fasting GIP level of 480 pg/ml which resulted in a mean fasting GIP concentration for the study group of 272 pg/ml. Fifteen minutes after the ingestion of corn oil, mean serum GIP increased to 417 ± 131 pg/ml; and within 30 min mean

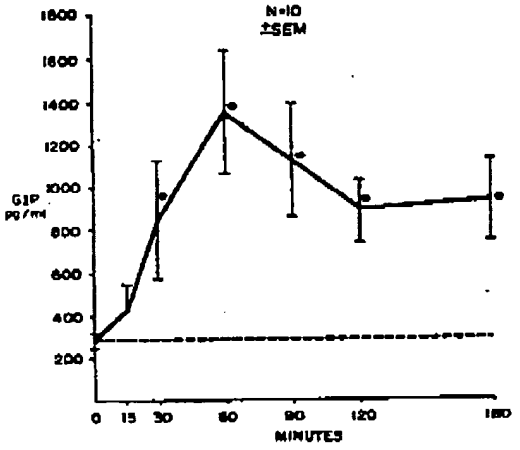


FIG. 1. Serum GIP responses in 10 normal subjects following the ingestion of 67 g of fat (solid line) compared to control studies (N = 6) (broken line) in which water was ingested. Values are mean serum GIP concentrations ± SEM. Asterisks indicate significant elevations over fasting values.

TABLE 1. Serum GIP concentrations (pg/ml) following Lipomul or water ingestion

		Time (min)					
	Fasting	15	30	60	90	120	180
Lipomul (n = 10)	272 ± 44	417 ± 131 NS†	856 ± 272 $P < 0.05$†	1,345 ± 291 $P < 0.005$†	1,117 ± 265 $P < 0.005$†	879 ± 142 $P < 0.005$†	937 ± 188 $P < 0.005$†
Water (n = 6)	273 ± 42	258 ± 14 NS	258 ± 14 NS	250* NS	250* NS	250* NS	250* NS

† Compared to fasting values. NS = not significant.
* All values at or below 250 pg/ml.

TABLE 2. Glucose, insulin and nonesterified fatty acid (NEFA) responses to 67 g of orally administered corn oil in 10 normal subjects*

		Time (min)					
	Fasting	15	30	60	90	120	180
Glucose mg/100 ml	73 ± 3	76 ± 2	73 ± 3	72 ± 2	72 ± 2	71 ± 2	71 ± 2
Insulin μU/ml	16 ± 2	19 ± 3	19 ± 3	18 ± 2	18 ± 2	17 ± 2	18 ± 2
NEFA μeq/liter	881 ± 63	881 ± 61	867 ± 50	828 ± 35	856 ± 54	936 ± 69	1,009 ± 45

* All data are mean ± SEM.

GIP concentrations were 856 ± 272 pg/ml, which represented a significant ($P < 0.05$) elevation over fasting levels. The peak mean serum GIP concentration of 1,345 ± 291 pg/ml occurred at 60 minutes, and although declining somewhat, the mean GIP levels remained significantly higher than fasting ($P < 0.005$) between 60 and 180 min.

Summarized in Table 2 are the glucose, IRI and NEFA concentrations after corn oil ingestion in these normal subjects. No significant changes in glucose, IRI or NEFA were observed during the time intervals studied following fat ingestion.

In Fig. 2, the GIP and insulin responses after fat ingestion are compared to those previously reported from this laboratory (4) after 75 g of oral glucose was given to normal volunteers. The peak serum GIP concentration observed after 67 g of corn oil (1,345 ± 291 pg/ml) is significantly higher ($P < 0.05$) than that observed after 75 g of oral glucose (747 ± 59 pg/ml). The GIP response was also more sustained after corn oil, being significantly greater ($P < 0.05$) between 60 and 180 min than after oral glucose (Fig. 2).

Discussion

In 1886, Ewald and Boas (11) observed that the addition of olive oil to a test meal given to human subjects depressed acid secretion. Subsequently, studies in Pavlov's laboratory showed that fat must reach the duodenum to exert this effect (12). Other investigators (13,14) have shown that the intraduodenal instillation of fat also inhibits the motor activity of a transplanted fundic pouch. In 1930, Kosaka and Lim, (2) after demonstrating that crude intestinal extracts inhibited gastric secretion, coined the term enterogastrone to

describe the active principle of the upper intestinal mucosa which was liberated by fat or its digestion products and inhibited gastric secretion and motility. However, the term enterogastrone is often used by other investigators (15,16) in a more general sense to designate gastric inhibitors released not only by fat, but also by acid and hyperosmotic solutions. Although a variety of candidate hormones have been suggested to have enterogastrone activity (15,16), GIP appears to satisfy most completely the criteria for being the enterogastrone described by Kosaka and Lim (2).

GIP is released by fat; and purified porcine GIP inhibits canine gastric acid secretion stimulated by pentagastrin, histamine and insulin hypoglycemia (17). Porcine GIP also inhibits canine pepsin secretion stimulated by insulin hypoglycemia and inhibits motor activity in pouches of the antrum and body of the stomach. These responses are observed with relatively small doses of intravenously administered porcine GIP ranging from 0.25 to 1.0 μg/kg/h (17).

Polak *et al.* (18), using an antiserum to purified porcine GIP, have identified this polypeptide by direct immunofluorescence in cells, tentatively identified as D_1 cells, in the duodenum and, to a lesser extent, in the jejunum in man and dog. Studies done in our laboratory by O'Dorisio *et al.* (19) have demonstrated that the highest concentrations of immunoassayable GIP in canine gastrointestinal tissue extracts are in the second and third portion of the duodenum. The concentration of GIP is almost as great in the jejunum and is lowest in the ileum and antral portion of the stomach. Initial observations in our laboratory suggest that a similar pattern of immunoassayable GIP is present in extracts of human gastrointestinal tissue.

If endogenous GIP has a physiologic enterogasterone effect in man, then its secretion should be stimulated by the ingestion of fat. This study demonstrates that oral fat is a potent stimulus to the secretion

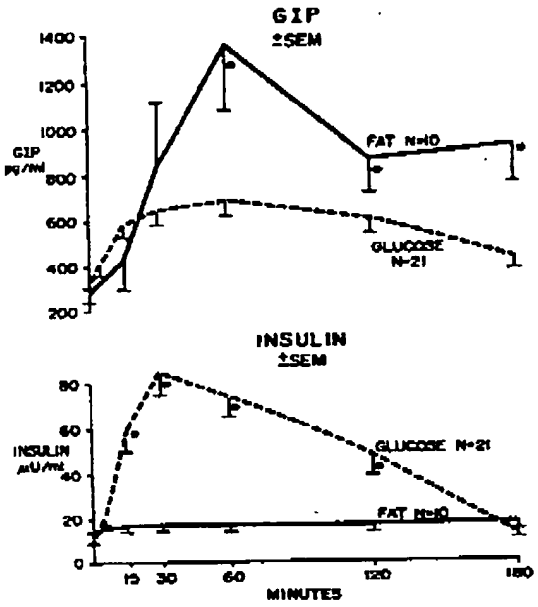


FIG. 2. Serum GIP and IRI responses to corn oil (solid line) compared to those previously reported (4) in response to 75 g of oral glucose (broken line). Values expressed as mean ± SEM. Asterisks indicate significant differences between GIP or IRI responses when those who ingested fat are compared to subjects given oral glucose.

of GIP in normal individuals. The serum concentrations of GIP which we observed are almost twice as high as those noted by Brown (6) in a preliminary report of a similar study done in several normal volunteers. The reason for this difference is not apparent. Dupré *et al.* (3) in a study of the insulinotropic effect of porcine GIP in man, infused 30 μg of GIP over 30 min which elevated immunoassayable GIP by approximately 1 ng/ml. Cleator and Brown (1) recently indicated that pure porcine GIP will inhibit acid secretion in man when administered in a dose of 2.0 μg/kg/30 min. This should raise immunoassayable GIP concentrations to approximately 3 to 4 ng/ml, a level higher than that observed in our volunteers after the ingestion of fat. However, GIP levels remained elevated in the range of 1 ng/ml for 3 h after fat ingestion, which is a somewhat longer

hypoglycemia, a phenomenon not ordinarily observed in man after the ingestion of fat. The results of our study indicate that endogenously released GIP is not insulinotropic in the absence of hyperglycemia, even at serum GIP concentrations in excess of those observed after a 75 g oral glucose stimulus. However, preliminary observations in our laboratory indicate that, in normal volunteers, the insulin response to *intravenous* glucose is augmented by the simultaneous oral ingestion of fat.

How GIP release is stimulated by fat is not answered by these studies and is the subject of continuing investigation. Much more evidence is necessary before a hormonal status can be ascribed to GIP or before it can be classified as both an enterogastrone and an insulinotropic hormone.

Acknowledgments

This study was supported in part by the Bremer Foundation 521532-7308, the National Institute of Arthritis, Metabolism and Digestive Diseases 5T01-AM 5118-18, and General Clinical Research Centers Program, National Institutes of Health RR-34. We wish to thank Dr. John C. Brown for the generous supply of antisera and purified GIP.

References

1. Brown, J. C., *Med Clin N Am* 58: 1347, 1974.
2. Kosaka, T., and R. K. S. Lim, *Chinese J Physiol* 4: 213, 1930.
3. Dupré, J., S. A. Ross, D. Watson, and J. C. Brown, *J Clin Endocrinol Metab* 37: 826, 1973.
4. Cataland, S., S. E. Crockett, J. C. Brown, and E. L. Mazzaferri, *J Clin Endocrinol Metab* 39: 223, 1974.
5. Kuzio, M., J. R. Dryburgh, K. Malloy, and J. C. Brown, *Gastroenterology* 66: 357, 1974.
6. Brown, J. C., J. R. Dryburgh, and R. A. Pederson, *In* Chey W. Y., and F. P. Brooks (eds.), Endocrinology of the Gut, Charles B. Slack, Thorofare, New Jersey, 1974, p. 80.
7. Kipnis, D. M., *Adv Intern Med* 16: 103, 1970.
8. Bierman, E. L., V. P. Dole, and T. N. Roberts, *Diabetes* 6: 475, 1957.
9. Velasco, C. A., W. Opperman, and R. A. Camerini-Davados, *Clin Chem* 19: 201, 1973.
10. Hunter, W. M., and F. C. Greenwood, *Nature* 194: 495, 1962.
11. Ewald, C. A. and J. Boas, *Virchows Arch* 104: 271, 1886.
12. Pavlov, I. P., The Work of the Digestive Glands, ed. 2, Translated by W. H. Thompson, Charles Griffin, London, 1910.
13. Farrell, J. I. and A. C. Ivy, *Am J Physiol* 76: 227, 1926.
14. Feng, T. P., H. C. Hou, and R. K. S. Lim, *Chinese J Physiol* 3: 371, 1929.
15. Grossman, M. I., *Gastroenterology* 67: 730, 1974.
16. Johnson, L. R., and M. I. Grossman, *Gastroenterology* 60: 120, 1971.
17. Pederson, R. A., and J. C. Brown, *Gastroenterology* 62: 393, 1972.
18. Polak, J. M., S. R. Bloom, M. Kuzio, J. C. Brown, and A. G. E. Pearse, *Gut* 14: 284, 1973.
19. O'Dorisio, T. M., S. E. Crockett, E. L. Mazzaferri, K. Sirinek, N. Thomford, and S. Cataland, *Clin Res* 22: 605A, 1974 (Abstract).
20. Thomas, F. B., E. L. Mazzaferri, S. E. Crockett, H. S. Mekhjian, H. D. Gruemer, and S. Cataland, *Clin Res* 23: 258A, 1975.
21. Sirinek, K. R., S. E. Crockett, E. L. Mazzaferri, S. Cataland, and N. R. Thomford, *Surg Forum* 25: 361, 1974
22. Moberg, S., and C. Carlberger, *J. Scan Gastroenterology* 9: 29, 1974.